

**INVESTIGATION OF STRATEGIES TO PROTECT
AGAINST HARMFUL BACTERIA – MUCOSA
INTERACTION IN CROHN’S DISEASE AND OTHER
DIARRHOEAL DISEASES**

**Thesis submitted in accordance with the requirements of the
University of Liverpool for the degree of Doctor of Philosophy by**

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DECLARATION

All techniques and experiments performed and described in this thesis were undertaken by myself as a PhD student at the University of Liverpool between March 2008 and January 2011, with the exception of ETEC and *C.difficile* FACS studies, which I performed with Dr Helen Martin and Monica Barclay, the ETEC and banana plantain fibre adhesion assay specifically I performed in collaboration with Dr Carol Roberts, and I initially performed plasmid work under the guidance of Dr Chloe James and Dr Joanne Fothergill of the Department of Clinical Infection, Microbiology and Immunology. Also, Dr Fei Song analysed some remission biopsy qPCR samples after I had set up the optimised qPCR technology for quantification.

Neither this thesis nor any part of it has been submitted in support of an application of another degree or qualification of this or any other University or other institute of learning.

Paul Philip Knight, December 2011

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SUPPORTING PAPERS

The work involved in this thesis has led to the following publications:

- 1) Knight P, Campbell BJ, Rhodes JM. Host-bacteria interaction in inflammatory bowel disease. *Br Med Bull* 2008; 88 (1):95-113.
- 2) P Knight, C. Winstanley, CL Roberts, BJ Campbell, JM Rhodes. The role of *Escherichia coli* in Crohn's Disease pathogenesis. *Proceedings of the XVI "Miquel A. Gassull" Course on IBD* June 2009.
- 3) Knight P, Winstanley C, Campbell BJ, JM Rhodes. Impact of drugs used in Crohn's disease on survival of *E. coli* within macrophages (In preparation).
- 4) Carol L Roberts;Åsa V Keita;Bryony N Parsons;Maelle Prorok-Hamon;Paul Knight;Craig Winstanley;Niamh O'Kennedy;Johan D Söderholm; Jonathan M Rhodes;Barry J Campbell. Soluble plantain fibre blocks adhesion and M-cell translocation of intestinal pathogens (Accepted by Journal of Nutritional Biochemistry).

SCIENTIFIC ABSTRACTS AND PRESENTATIONS

Plantain fibres inhibit translocation of Crohn's Disease *E. coli* Isolates and Intestinal Pathogenic bacteria across M-cells. Maelle Prorok-Hamon, Carol Roberts, Melissa Friswell, Helen Martin, Paul Knight. Poster presentation for the University of Liverpool, 2009.

***Escherichia coli* in Crohn's Disease and diarrhoea-causing pathogens: adherence and inhibition. Translating from laboratory to bedside.** Paul Knight, Carol Roberts, Maelle Prorok-Hamon, Niamh O' Kennedy, Barry Campbell, Sree Subramanian, C Anthony Hart, Craig Winstanley and Jonathan Rhodes. Poster Presentation for the opening of the Biomedical Research Centre (BRC) Clinical Research Facility at the Royal Liverpool University Hospital, 2009.

Novel treatments for Crohn's disease and diarrhoeal illness. Paul Knight, Barry Campbell, and Jonathan Rhodes, Poster presentation, University of Liverpool, 2009.

Evidence against intra-macrophage replication of Crohn's Disease *E.coli* being dependent on an anaerobic environment within the phagolysosome – lack of efficacy for metronidazole. Paul Knight, Barry Campbell, and Jonathan Rhodes. Poster Presentation at BSG, Liverpool 2010.

Gut 59:A98 doi:10.1136/gut.2009.208967d

Azathioprine and Hydrocortisone but not Metronidazole inhibit intra-macrophage replication of Crohn's disease *E. coli* Paul Knight, Barry Campbell, Craig Winstanley, and Jonathan Rhodes. Poster Presentation at Northwest microbiology meeting, Liverpool, 2010.

Azathioprine and Hydrocortisone but not Metronidazole inhibit intra-macrophage replication of Crohn's disease *E. coli*. Paul Knight, Barry Campbell, Craig Winstanley, and Jonathan Rhodes. Poster presentation at UEGW, Barcelona, 2010. *Gut* 2010; **59** (Suppl III) A288, P0899

Azathioprine and 6-Thioguanine but not 6-Mercaptopurine inhibit intra-macrophage replication of Crohn's disease *Escherichia coli*. Paul Knight, Barry Campbell, Craig Winstanley, and Jonathan Rhodes. Poster presentation at BSG, Birmingham, 2011.

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Soluble plantain fibre blocks epithelial adhesion and M-cell translocation of intestinal pathogens. Carol L. Roberts, Åsa V. Keita, Bryony N. Parsons, Maelle Prorok-Hamon, Paul Knight, Niamh O'Kennedy, Johan D Söderholm, Jonathan M. Rhodes, and Barry J. Campbell. Poster presentation at the BSG, Birmingham, 2011.

Gut 2011;**60**:A96 doi:10.1136/gut.2011.239301.198

Soluble plantain fibre blocks epithelial adhesion and M-cell translocation of intestinal pathogens. Carol L. Roberts, Åsa V. Keita, Bryony N. Parsons, Maelle Prorok-Hamon, Paul Knight, Niamh O’Kennedy, Johan D Söderholm, Jonathan M. Rhodes and Barry Campbell. Poster presentation at DDW, Chicago, 2011.

Gastroenterology; **140**:5, Supplement 1, Page S-362, DOI: 10.1016/S0016-5085(11)61479-0) Sa1987

Evaluation of novel therapies targeting adhesive/invasive *E. coli*. Paul Knight, Barry J. Campbell, Craig Winstanley and Jonathan M. Rhodes. Presentation for the BRC Themes meeting, Liverpool, 2009.

***E. coli* in Crohn’s Disease pathogenesis.** Paul Knight, Barry J. Campbell, Craig Winstanley and Jonathan M. Rhodes. Young Researchers Talk, University of Liverpool, 2009.

LIST OF ABBREVIATIONS USED IN THIS THESIS

AC : ascending colon

AD : at diagnosis

ADA : adalumimab

ADP : adenosine diphosphate

A/E : adherent-effacement (lesion)

AIEC : adherent invasive *E. coli*

AK : adenylate kinase

Amp X-gal : ampicillin X-gal (5-bromo-indolyl-beta-D-galactopyranoside)

AP : acute pancreatitis

AS : aphthous stomatitis

5 - ASA : 5-aminosalicylic acid

ASCA : anti-*Saccharomyces cerevisiae* antibody

ATG16L1 : ATG16 autophagy related 16 like 1

ARISA : Automated ribosomal intergenic spacer analysis

AU : aphthous ulcer

Aza : azathioprine

BCECF/AM : 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester

bfp : bundle-forming pili

BLAST : basic local alignment search tool

bp : base pair(s)

BSA : bovine serum albumin

CARD 15 : caspase recruitment domain 15

CCR6 : (cysteine-cysteine) chemokine receptor 6

CD : Crohn's disease

CDAI : Crohn's disease activity index

CEACAM : Carcino-embryonic antigen-associated cell-adhesion molecule

CFA : colonisation factor antigen

CFU : colony forming unit(s)

C_{max} : peak serum concentration of drug achievable using a conventional dosing regimen

CONT : control

C_q : quantification cycle (also C_T or C_p)

C_p : crossing point

CRF : corticotrophin releasing factors

CTZ : certolizimab

CV : coefficient of variation

DEP : diarrhoeagenic *E coli* pathotypes

DGGE : denaturing gradient gel electrophoresis

DMEM : Dulbecco's modified Eagle medium

DMSO : dimethyl sulfoxide

DNA : deoxyribonucleic acid

DsbA : disulphide bond forming protein A

DTT : dithiothreitol

DZ : dizygotic

EAF : EPEC-adherence factor

EHEC : entero-haemorrhagic *E. coli*

EIM : extra-intestinal manifestations

ELISA : enzyme-linked immunosorbent assay

EN : erythema nodosum

EPEC : entero-pathogenic *E.coli*

ETEC : entero-toxigenic *E.coli*

EudraCT : European clinical trials database

FACS : fluorescence-activated cell sorting

FAE : follicle associated epithelium

FBS : fetal bovine serum

FCS : fetal calf serum

FDR : first degree relative

FISH : fluorescence *in situ* hybridization

Fprau : *Faecalibacterium prausnitzii*

g : acceleration due to gravity

GMPS : guanine monophosphate synthetase

GTP : guanosine triphosphate

GWAS : Genome Wide Association Scan

H and E : haematoxylin and eosin

HBI : Harvey-Bradshaw Index

HC : hydrocortisone

HLA : human lymphocyte antigen

HM : Helen Martin

HPRT : hypoxanthine guanine phosphoribosyl transferase

HtrA : ‘high temperature requirement’ protein A

IBD : inflammatory bowel disease

IFX : infliximab

IL-8 : interleukin 8

IL-23R : interleukin 23 receptor

IMPDH : inosine-5-monophosphate dehydrogenase

iNOS : inducible nitric oxide synthetase

IP₃ : inositol triphosphate

IPTG : isopropyl-1-thio-β-D-galactopyranoside.

IRGM : immunity related guanosine triphosphatase

IS 900 : insertion sequence 900 (of MAP)

ISRCTN : international standard randomised control trial number

ITPase : inosine triphosphatase

iucD : iron uptake chelate

IV : intravenous

LB : Luria Bertani

LEE : locus for enterocyte effacement

LF : lymphoid follicle

LI : large intestine

LNA : locked nucleic acid

LOD : limit of detection

lpf : long polar fimbriae

LRR : leucine rich region

LT : heat labile (toxin)

MAP : *Mycobacterium avium paratuberculosis*

Mbp : mega base pairs

m-cells : microfold cells

MDP : muramyl dipeptide, MurNAc-L-Ala-D-isoGln

MDR : multi-drug resistance

MFI : mean fluorescent intensity

6-MMP : 6-methymercaptopurine

6-MMPR : 6-methylmercaptopurine ribonucleotides

MMR : Measles, mumps and rubella

MOI : multiplicity of infection

6-MP : 6-mercaptopurine

MST-1 : macrophage stimulating 1

MUC : mucin

mv : microvillous

MyD88 : Myeloid differentiation factor 88

MZ : monozygotic

N_A : Avogadro's number

NAC : non amplification control

NCBI : National Center for biotechnology information

ND : not detected

NF κ B : nuclear factor kappa B

NOD2 : nucleotide oligomerisation domain 2

NOS : Nitric Oxide Synthetase

NSP : non-starch polysaccharide

NTC : non template control

NZY : nizatidium bromide

OD : optical density

Oligonucleotide : a short polymer of nucleic acids

PAMP : pathogen-associated molecular patterns

pANCA : perinuclear anti-neutrophil cytoplasmic antibody

PCR : polymerase chain reaction

PG : pyoderma gangrenosum

PBS : phosphate-buffered saline

PKC : protein kinase C

PLC : phospholipase C

PRR : pattern recognition receptors

PSC : primary sclerosing cholangitis

qPCR : quantitative polymerase chain reaction

RCT : randomised controlled trials

rDNA : ribosomal DNA

RefSeq : reference sequence

REL : relapse

RISA : ribosomal intergenic spacer analysis

RLBUHT : Royal Liverpool and Broadgreen University Hospital NHS Trust

RMM : relative molecular mass

RNA : ribonucleic acid

ROS : reactive oxygen species

RPM : revolutions per minute

RPMI : Roswell Park Memorial Institute medium

RR : relative risk

rRNA : ribosomal RNA

RT : real time

SC : sigmoid colon

SD : standard deviation

SEM : standard error of the mean

Serp : serpingous

SF : splenic flexure

SI : small intestine

S.O.C. : super optimal broth with catabolite repression (or enriched bacterial growth medium)

SPATES : serine protease autotransporters

ST : heat stable (toxin)

TAE : Tris-acetate-EDTA

Taq : thermus aquaticus

TBE : Tris-Borate-EDTA

6-TG : 6-thioguanine

6-TGN : thioguanine nucleotides

TGF : transforming growth factor

Th : T helper

6-thio-ITP : 6-thio-inosine triphosphate

TI : terminal ileum

6-TIMP : 6-thioinosine monophosphate

Tir : translocated intimin receptor

TLR : toll-like receptors

TNF α : Tumour necrosis factor α

TPMT : thiopurine *S*-methyltransferase

TREAT : The Crohn's Therapy, Resource, Evaluation, and Assessment Tool Registry

T-RFLP : terminal restriction fragment length polymorphisms

triDAP : L-Ala- γ -D-Glu-*meso*-diaminopimelic acid

6-TU : 6-thiouric acid

UC : ulcerative colitis

UPEC : uropathogenic *E.coli*

UV : ultraviolet

v/v : volume per volume

WDL : withdrawal

Wnt : 'wingless type'

w/v : weight per volume

X-Gal : 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

XO : xanthine oxidase

ABSTRACT

The presence and replication of *E.coli* within Crohn's Disease (CD) tissue has been confirmed by multiple authors and is hypothesised to be pivotal in the development of CD. In this thesis, quantification of *E.coli* bacteria within endoscopic biopsies has been achieved with high efficiency, sensitivity and reproducibility using PCR plasmid technology, and the technique's utility demonstrated by quantifying *E.coli* within the biopsies of CD patients in clinical relapse and remission as well as within the first lesions present in CD relapse, aphthous ulcers. These studies showed some interesting correlations with clinical, macroscopic, and histological data, obtained during a clinical trial of soluble plantain fibre supplementation for prevention of relapse in CD. These included increased quantities of *E.coli* in tissues from the mucosa of patients in clinical relapse whose ileum was macroscopically normal yet histologically inflamed, and significant falls in the quantities of *E.coli* over time in the biopsy tissues of patients who were in clinical remission.

The ability of a specific group of *E.coli*, the adherent invasive *E. coli* (AIEC), to replicate within macrophages, is increasingly perceived to be fundamental in CD pathogenesis. The replication of *E.coli* within the phagolysosomes of macrophages is implicated in the release of pro-inflammatory cytokines, granuloma formation, and the consequent mucosal injury seen in CD. The pharmacological inhibition of AIEC replication in this work within murine macrophage tissue *in vitro* by the immunosuppressive azathioprine and its active metabolite 6-thioguanine as well as by the antibiotic ciprofloxacin at clinically relevant concentrations is supportive of this central hypothesis, and also suggests new treatment combinations that might be trialled in active CD. Interestingly and unexpectedly the steroid hydrocortisone also inhibited AIEC replication within macrophages, suggesting that sepsis-related complications seen with steroid use clinically may be related to its effects on neutrophil recruitment, rather than on phagocytic killing of bacteria.

The diarrhoeal pathogens EPEC, ETEC, and *C. difficile* represent a large disease burden in terms of infantile, travellers', and antibiotic-associated diarrhoea respectively with *C. difficile* also sometimes implicated in the exacerbation of CD. Plantain (banana) non starch polysaccharide (NSP) has previously been demonstrated to inhibit AIEC adhesion to mucosal tissues, and this work demonstrates the inhibition of the adherence of ETEC and *C. difficile* to intestinal cells *in vitro* with two different modalities, using concentrations of soluble plantain fibre that are readily achievable in the distal intestinal lumen. Previous work on AIEC inhibition with plantain was confirmed, whilst oat fibre and apple pectin did not significantly inhibit ETEC adherence. EPEC was not inhibited by plantain fibre, which may be due to its unique epithelial interaction. These foodstuffs, if prepared as suitable supplements, may offer new prophylactic therapeutic interventions for global and institutional diarrhoeal illness following appropriate clinical trials.

CHAPTER ONE:

INFLAMMATORY BOWEL DISEASE

1.1 Natural History of Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) comprises mainly Crohn's disease (CD) and ulcerative colitis (UC). These diseases affect mainly the small (CD) and large (CD and UC) intestines, and their pathogenesis involves genetic and environmental factors that include alterations in innate immunity, the function of the mucosal barrier and the intestinal microbiota (1).

1.1.1 CD

CD is characterised by deep inflammation with granulomata, and may affect any part of the gastrointestinal tract, although it most commonly affects the distal ileum and caecum. CD may affect the buccal cavity (9%) with inflammation causing aphthous or granulomatous ulcers as well as disfiguring infiltration of the lips that is commoner in children with CD, and precedes the development of intestinal symptoms in 60% of patients (2). The oesophagus may less commonly be affected (3). Gastroduodenal inflammation has been reported in 20% of CD endoscopy cases, mainly as *H. Pylori* negative focal gastritis with 40% involvement histologically but with less than 4% clinically symptomatic from these lesions (4). Duodenal involvement is commoner in younger patients and may be particularly troublesome

with upper GI stricturing (5). Rarely high fistulae complicate CD with 5 cologastric (0.6 %) and 1 ileogastric (0.08 %) fistulae seen in a series of cases over 30 years at Mount Sinai Hospital, New York (6). Acute Pancreatitis (AP) is a rare extra-intestinal complication of IBD, but much more commonly precedes IBD diagnosis in children than adults with one retrospective analysis involving 3960 patients showing a rate of 2.17% AP preceding IBD in children versus 0.06% in adults (7). Another retrospective analysis of 230 children demonstrated 30% having gastric, oesophageal, or duodenal involvement, with 3 patients having CD confined to the upper gastrointestinal tract (1.3%). It was also observed that children were significantly (33%) more likely to have upper gastrointestinal pathology if they had both large and small intestinal CD involvement compared to either small or large intestinal disease in isolation (8). Diffuse small intestinal disease, jejunoileitis, is difficult to treat, but is uncommon with its exact incidence unknown, though most gastroenterologists feel its prevalence is increasing, and again it is more frequently seen in patients presenting in childhood (see table 1.2, figure 1.1, and (9). Disease is commonly seen in the terminal ileum (16-25%) and proximal colon, with ileocolonic disease present in 37-41%. Isolated colonic disease occurs in about 1/3 to 1/2 of cases (35-47%)(10), but here distinction from UC may be difficult (see later). The commonest site of involvement in CD varies with ethnicity and for south Asians living in London is predominantly colonic (46.8%), compared with the higher prevalence of ileocolonic disease (40.5%) in North European north London inhabitants (see table 1.1). Lesions in CD are typically discontinuous, frequently spare the rectum and can give a characteristic 'cobblestone' appearance. The colonic aphthous ulcer is a lesion seen in early active CD (11) and is highly suggestive of the diagnosis.

Fistulae in CD in general have a cumulative incidence of 33% 10 years after diagnosis, and 50% 20 years after diagnosis (12), whilst peri-anal disease including fistulae (abnormal connections between two epithelial surfaces), fissures, and abscesses affects 25-80 % of patients with CD (13), and is an independent predictor of change in CD from an initial non-stricturing non-perforating behaviour to other disease phenotypes (14), & table 1.3.

Table 1.1 Prevalence of pathology in CD by location and surgery risk

*** = statistics from paediatric cohorts only

** = predicts development of complicated disease and surgery

* = 50% combined risk of resection during median 7 year follow up (mixed cohort)

RR = relative risk AD = at diagnosis (References (3-10;12;13;15)

Location of CD	%, or % range affected	Long term prognosis/risk for surgery
Isolated Ileal disease	16-24.7	0.84% resection AD. Significantly greater risk than colonic pathology of complicated disease * and **
Isolated Colonic disease	34.8–46.8	0.63% resection AD *
Ileocolonic disease	37.2-40.5	6.9% resection AD * and ** 25-91% reoperation risk after 15y ***
Gastroduodenal	4-40	** (for duodenal involvement)
Oesophageal, gastric or duodenal	30 ***	** (for duodenal involvement)
Strictureing	>26	** 28-44% recurrence after stricturoplasty (9y follow up) ***
Upper GI Fistulating	0.08-0.6	** 89% had surgery for this finding
Pancreatitis	0.06-2.17	
Mouth (Aphthoid) ulcers	9	
Perianal disease	25-80	Ileal and perianal disease have RR of fistulation of 0.8-2.2. colonic and perianal disease have RR for fistulae of 3.4
Cumulative incidence of fistulae in CD	33 (10 years) 50 (20 years)	

Table 1.2 Early onset CD phenotype (from (9)).

	<20 Years	20–39 Years	>40 Years
Family history (%)	30	19	14
Ileal involvement (%)	89	78	58
Colonic involvement (%)	70	65	85
Strictures (%)	46	34	29
Inflammation (%)	34	45	55
Surgery (%)	71	—	56

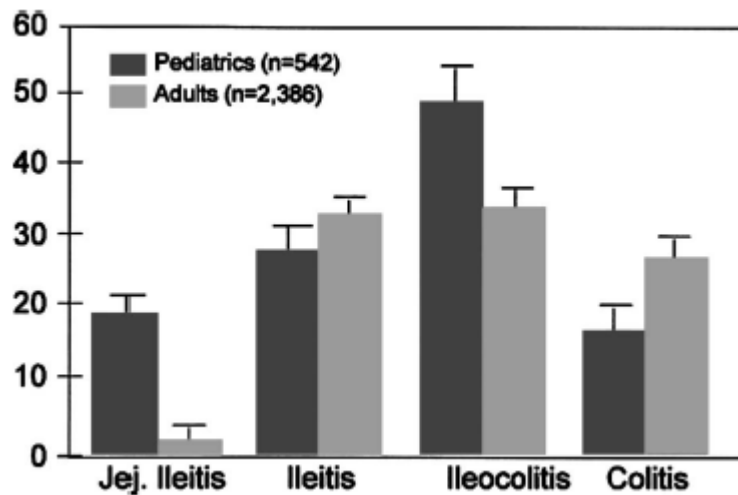


Figure 1.1 Site of CD in children and adults, from (9).

1.1.1.1 The Vienna classification of CD

The Vienna classification of CD (16) was an attempt to put CD cases into more homogenous groups with prediction of disease behaviour based on the variables age of diagnosis and location of disease (see table 1.3). Cross table analysis of these cases shows association between age of diagnosis and location of disease as well as

between location of disease and behaviour. Such a classification is useful as it can predict requirements for either medical, endoscopic or surgical assessment and treatment, and guide patient counselling. The Vienna classification (16) divides CD behaviour into penetrating, stricturing and inflammatory disease. Stricturing is narrowing of the bowel lumen which can lead to bowel obstruction, and penetrating disease implies fistulae between the skin and bowel or bowel and other organs or epithelia in the abdomen and pelvis. Inflammatory disease reflects absence of stricturing or fistulae.

Table 1.3 The Vienna classification of CD, modified from (16)

1 = the age when the diagnosis of CD was first definitively established by endoscopy, radiology, surgery or pathology, **2** = the maximum extent of disease involvement at any time prior to the first resection, minimum involvement for a location is defined as any aphthous lesion or ulceration. Mucosal erythema and oedema are insufficient. For classification at least both a small bowel and a large bowel examination, are required. **3** = Disease limited to the terminal ileum (the lower third of the small bowel) with or without spill over into cecum. **4** = Any colonic location between cecum and rectum with no small bowel or upper gastrointestinal (GI) involvement. **5** = Disease of the terminal ileum with or without spill over into cecum and any location between ascending colon and rectum. **6** = Any disease location proximal to the terminal ileum (excluding the mouth) regardless of additional involvement of the terminal ileum or colon. **7** = Inflammatory disease which never has been complicated at any time in the course of disease. **8** = Stricturing (or stenosing) disease is defined as the occurrence of constant luminal narrowing demonstrated by radiologic, endoscopic or surgical-pathologic methods with prestenotic dilatation or obstructive signs/symptoms without presence of penetrating disease at any time in the course of disease. **9** = Penetrating (or perforating) disease is defined as the occurrence of intra-abdominal or perianal fistulas, inflammatory masses and/or abscesses at any time in the course of disease. Perianal ulcers are also included. Excluded are postoperative intra-abdominal complications and perianal skintags.

Age at diagnosis: ¹	A1, < 40 years
	A2, ≥ 40 years
Location: ²	L1, Terminal ileum ³
	L2, Colon ⁴
	L3, Ileocolon ⁵
	L4, Upper GI ⁶
Behaviour:	B1, Nonstricturing nonpenetrating ⁷
	B2, Stricturing ⁸
	B3, Penetrating ⁹

Applying the Vienna Classification system retrospectively to some cohorts has produced varied results. In one cohort it has been shown that while CD location remains relatively unchanged over time following diagnosis, with only 16% changing location, behaviour can change dramatically (up to 46%), with another cohort showing 91% stability of location. Other factors, such as perianal disease can have a large effect on CD behaviour change. The most common change in behaviour was from non-stricturing and non-penetrating to stricturing (27%) and penetrating (29%) after 10 years, in ileal and colonic or ileocolonic CD respectively. In this cohort of 125 patients examined, interestingly, and at some odds with the Vienna classification age at diagnosis had no impact on disease location or behaviour (17;18).

The Montreal revision of this classification examined serological and molecular classification of CD (19), and separated upper gastro-intestinal disease and perianal disease as different CD entities with different natural histories.

1.1.1.2 Factors affecting the progression rate of CD

Factors that can affect progression rate of CD include smoking, peri-anal disease, prior steroids, and early onset of immunosuppression (14). Other studies identify weight loss and steroid requirement at first presentation (20) as prognostic risks. Perianal disease in colonic CD has significant risk of progressing to cause internal fistulisation (21) and small bowel location, ileo-colonic CD (20) and stricturing predict progression to complicated disease and surgery (17;22). Conversely, disease extension and severity can be modified by use of the immunomodulator, azathioprine (23;24). Early thiopurine use has been associated

with lower cumulative surgical resection rates in CD over time (25). Environmental factors affecting disease are discussed in more detail later.

Independent of the classification system and treatments used, it is clear that CD is dynamic and can evolve, particularly from a non-stricturing, non-penetrating form into these more complicated disease forms, which is dependent on genotype and environmental exposures.

In general most CD patients have a relapsing remitting course of illness, with 13% having unremitting disease and 10% having more prolonged remission, and overall in an analysis of North American patients there was up to a 57% chance of at least one surgical resection during the course of the disease (26).

1.1.2 Ulcerative Colitis

Ulcerative colitis classification is much simpler than that of CD, with three groups identified: proctitis or proctosigmoiditis (affecting rectum and sigmoid colon), “left sided colitis” (up to the splenic flexure) and pancolitis (inflammation affecting the whole bowel).

UC is largely limited to the colon, particularly the distal colon and rectum and causes more superficial ulceration than CD (27). A large cohort of IBD patients were evaluated in London, and 63% of the South Asian population there had extensive colitis “pancolitis”, with the figure for North Europeans living in London at 42.5%. The prevalence of left sided UC was 27.1% and 31.4% respectively for the South Asian and North European communities, with cases having disease limited to the rectum “ulcerative proctitis”, at 9.9% and 26.1% respectively with higher prevalence of this localised disease seen in the North European group (10). In a study of 1116

patients in Cleveland prevalence was 36.7% for pancolitis, 46.2% for proctosigmoiditis, and 17% for left sided colitis, which is comparable to the London based cohort for pancolitis prevalence. The mean follow up was 12.7 years, but within 2 years 16.7% had colonic haemorrhage, with 12.7% having toxic colitis with a total surgical intervention rate of 37.6% over the follow up period. The chances of extension of disease are 70% in 5 years for left sided colitis, and 50% in 25 years for proctitis, with both initial and final disease location affecting colectomy and ileostomy rates. Factors which affected disease progression were younger age at diagnosis, extent of disease, toxic colitis, joint symptoms and severe haemorrhage (28). Age was independently associated with extent at diagnosis too, with younger age associated with a greater extent of mucosal damage (pancolitis), but not necessarily a worse disease course or increased rate of colectomy (29).

Histological findings in UC include mucosal inflammation which is continuous and circumferential and extends proximally from the rectum to involve the colon and in severe cases the terminal ileum “backwash ileitis” may be involved, though this may be a misnomer and represent a discrete disease entity whose incidence is not comprehensively quantified. In a series of 100 patients who had restorative proctocolectomy for UC, 22% of these had ileitis and this was strongly associated with pancolitis, as well as associated with short duration to presentation, primary sclerosing cholangitis (PSC), but not with severity of colitis (30).

1.2 Extra-intestinal manifestations of IBD

Both conditions can be associated with extra-intestinal manifestations (EIM) of disease (31), such as skin rashes, joint pain and eye inflammation. In a paediatric study of 1019 patients 28.2% were affected by at least one EIM, with 87% of these occurring in the first year of enrolment to the study, and 17% at baseline (see table 1.4). Increased baseline clinical disease activity was associated with at least one of: arthralgia, erythema nodosum (EN), and aphthous stomatitis (AS) during the period of follow up (26.2 months) for both UC and CD, though there were statistically different rates of EN, AS, and Primary Sclerosing Cholangitis (PSC) between the conditions. PSC did not appear to be related to disease activity, but was more common in UC than CD, while the converse was true for EN and AS. Females with UC were more likely to experience arthralgia, and older patients were more likely to suffer PSC. PSC activity may be independent of IBD activity, and is not associated with underlying IBD activity in adults either (32;33), Extent of CD was not associated with EIM differences, whilst a pancolitis was associated with greater chance of EIM than limited disease (34). Interestingly, patients treated with mesalamine, infliximab or immunomodulator therapy were less likely to have an EIM (IBD therapy discussed later).

Table 1.4 Extra-Intestinal Manifestations of IBD, and significant differences between disease groups from (34).

	Total	CD	UC
Total	1009	728	281
Any EIM ^a	285 (28.2%)	218 (29.9%)	67 (23.8%)
Arthralgia	166 (16.5%)	124 (17%)	42 (14.9%)
AS	81 (8%)	72 (9.9%)*	9 (3.2%)*
Arthritis	37 (3.7%)	32 (4.4%)	5 (1.8%)
EN	28 (2.8%)	26 (3.6%) [#]	2 (0.71%) [#]
PSC	15 (1.5%)	7 (1%) [^]	8 (2.8%) [^]
Pancreatitis	9 (0.9%)	5 (0.7%)	4 (1.4%)
Chronic active hepatitis	6 (0.6%)	2 (0.3%)	4 (1.4%)
I/U	7 (0.7%)	6 (0.8%)	1 (0.4%)
Ankylosing spondylitis	4 (0.4%)	4 (0.5%)	0
PG	3 (0.3%)	3 (0.4%)	0

^a = individual subjects ≥ 1 EIM. AS = aphthous stomatitis; CD = Crohn disease; EN = erythema nodosum; I/U = iritis/uveitis; PG = pyoderma gangrenosum; PSC = primary sclerosing cholangitis; UC = ulcerative colitis. * $P < 0.001$, [#] $P = 0.010$, [^] $P = 0.039$.

In 1976 a series of 700 patients explored EIM and their associations with different locations and levels of activity in IBD (35), identifying major EIM as mouth, skin, joint and eye related. These occurred with a total rate of 36% with skin disease in 23%, joint problems in 15%, and eye and mouth EIM in 4%. Liver disease, gallstones, renal stones, peptic ulcer and amyloid were also documented. Pyoderma gangrenosum (PG) was observed more commonly in UC and EN more commonly in “granulomatous colitis”.

1.2.1 Arthropathy

One in four paediatric patients has an EIM and a similar rate is seen with adult IBD patients at 25-40% (34). Musculoskeletal, arthritic associations with IBD are in the form of spondylarthropathies, and include axial symptoms, peripheral arthritis and ankylosing spondylitis in 3-6%, but with sacro-iliitis more commonly in 14-20% making it the most common form of EIM, though the mechanism of arthropathy is

still unknown (36). Peripheral findings include sero-negative spondyloarthritis, arthropathies, enthesopathies, and dactylitis in 11-20% (37). These statistics are for both UC and CD. The peripheral pauci-articular arthropathies are often associated with IBD intestinal activity, but axial back pain, sacro-ileitis and ankylosing spondylitis are not. Sometimes arthropathy can be the presenting problem of an IBD exacerbation, and even though treating mucosal inflammation effectively does not always completely resolve arthralgia, the arthropathies associated with underlying IBD activity, mainly reactive non-erosive asymmetrical arthritis, usually resolve when the underlying disease is treated, whilst ankylosing spondylitis does not. Anti-tissue necrosis factor (anti-TNF) therapies have great promise in this area (see later) (36).

1.2.2 Skin and Eye Involvement

In terms of skin manifestations, EN (4.0%) and PG (0.75%) are the most common skin complications in IBD. In a total cohort of 2402 patients with CD and UC, 5.8% had at least one skin manifestation. PG is a neutrophilic dermatosis, causing necrosis and pigmented ulcers on the legs. EN is a panniculitis, with tender nodules present on the shins. In the cohort studied, EN was associated with CD, PG and joint involvement, as well as colonic involvement. PG was associated with uninterrupted pancolitis, eye involvement and EN, but neither was associated with a mixed surgical and medical severity assessment corresponding to disease activity in the IBD cohort studied. However the lesions can predict phenotype to some degree (38).

Eye involvement in IBD occurs in approximately 3-10% of patients, mainly as episcleritis and uveitis (38). Episcleritis runs a course parallel to that of the underlying

intestinal activity, whilst uveitis does not, and hence episcleritis may respond to IBD treatment better (39).

1.3 Epidemiology of IBD

IBD tends to be a disease of young adulthood, but diagnosis age has bimodal peaks (15-35, and to a lesser extent 50-70 years). The diagnosis of UC tends to be later than CD (40;41) CD is more common in women than men, with UC slightly more common in men than women in a study of hospitalised patients. (42)

The incidence of IBD is rising rapidly in developed countries (43), with immigrants also seemingly acquiring increased risk. Developing countries disease rates are increasing with UC rate increases shortly followed by an increase in CD rate (44). These observations and the relative lack of concordance of disease in monozygotic twins (see later) are all suggestive of the role of environmental factors in pathogenesis.

The prevalence of CD in North America ranges from 26.0 to 198.5 per 100,000 persons with an incidence of 3.1-14.6 per 100,000 person-years. (26). Prevalence and incidence across the world varies with greater rates amongst whites and Ashkenazi Jews and in industrialised northern countries. (45) Originally, the incidence and prevalence of IBD was thought to be much higher in North America, northern Europe, and the United Kingdom with lower rates in Africa, Asia, Latin America, and southern Europe, but the differences are thought to be smaller now with changes in socio-economic status (46).

1.4 Prognosis

Determining the extent and severity of disease helps in prediction of disease course (17) and risk of surgery (see table 1.1 and section 1.5) but the lifetime risk for surgery remains disappointingly constant at around 25% for UC and 80% for CD (47). Current treatments only target the consequences of the disease and not the underlying pathogenic mechanisms. Better understanding of pathogenesis should lead to different, safer, and more effective therapies.

1.5 Genetic Factors in IBD – Familial Clustering and Twin Studies

A large number of studies have shown familial clustering in IBD, with informative twin studies and aggregation seen of specific phenotypes of disease. Approximately 25% of patients with IBD have another family member affected. Follow up of the first twin pair IBD register from 1988 showed remarkable concordance between twins for disease characteristics including phenotype using the Vienna classification. Age of diagnosis, progression of extent, location and behaviour were the disease characteristics assessed. 80 twin pairs were identified with at least one twin affected by IBD, in 16 monozygotic (MZ) twin pairs with UC, only 1/16 were concordant compared to 8/18 MZ twins with CD. In 46 dizygotic (DZ) twin pairs with IBD, only 1 pair was concordant (for CD) Pair concordance for MZ twins with CD was 50%, with a much lower rate for UC of 18.8%, and in a Danish cohort amongst Danish twins the concordance rate for CD and UC in MZ twins was 58.3% and 18.2% respectively with DZ twins correspondingly 0% and 4.5% (48) suggesting that CD has a stronger genetic component than UC, but also that an environmental component

is important in disease pathogenesis (49). IBD was also more common in the relatives of patients with UC or CD, with a general prevalence of 7.9%, and first degree relatives (FDR) of a UC sufferer had a 15 fold increased risk of UC than non relatives, and an increased chance of early onset of disease, as well as total colitis. Interestingly CD was 3.5 fold more likely in the FDR of a patient with UC too, supporting the view that these are not two completely discrete conditions, but perhaps a continuum (50). When familial clustering of CD was evaluated in 1048 patients, a similar pattern was seen with FDR of CD sufferers having a 13.4% prevalence of CD. In addition FDR had a 21 fold increased risk of CD, with a 6 fold increased risk of UC compared to unrelated patients (51). The same pattern was seen with a Danish cohort; FDR had 10 fold increased risk of UC or CD, with a particular association seen in those where the sufferer had a diagnosis before the age of 50 (52). As well as statistically increased IBD rates between relatives, the phenomenon of genetic anticipation is also seen in IBD, where the age at diagnosis is earlier in affected children than in their parents, with a greater effect seen in UC than CD (53).

IBD rates also vary with ethnicity and the FDR of patients with IBD show differences between groups. The age specific rates for IBD in the FDR of sufferers who were non-Jews in California were 5.2 and 1.6% for CD and UC respectively, compared with the consistently higher rates seen in the FDR of the Jewish community with IBD in California who had rates of 7.8 and 4.5% respectively (54).

1.5.1 Genetic associations with IBD – Genome Wide Association Scan (GWAS) studies

Along with the above familial clustering and twin studies, other large studies in man show that UC and CD are polygenic disorders whose candidate predisposing genes have been clarified by a genome wide study (55). Ninety-nine gene associations with IBD have been identified. The NOD2/CARD 15 (nucleotide oligomerisation domain 2/ caspase recruitment domain 15) gene has been confirmed as an innate immune system gene polymorphism associated with ileal (56) and stricturing phenotypic variants of CD. It probably accounts for approximately 15% of CD causation in Western countries although is not associated with CD in Japan. NOD receptors along with Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) for microbes (figure 1.2). NOD proteins are cytosolic whilst TLRs are membrane bound (57).

Other bacterial handling genes identified in the genome-wide scan (58), include ATG16L1 (ATG16 autophagy related 16 like 1) gene, and IRGM (immunity related guanosine triphosphatase); a gene encoding a GTP binding protein which induces autophagy. Autophagy is involved in killing of intracellular bacteria including *Mycobacterium tuberculosis* (55). MST-1 (macrophage stimulating 1), also associated with CD, encodes a protein that influences motile activity and phagocytosis by peritoneal macrophages (55).

Associations with mutations in the interleukin 23 receptor IL-23R, have also been reproducibly found (55). IL-23R encodes a subunit of the receptor for the pro-inflammatory cytokine IL-23, which is pivotal in the differentiation of T helper

(Th17) cells. In animal models the Th17 T cell subset mediates chronic and autoimmune inflammatory conditions. (59)

Most of these associations, apart from NOD2/CARD15, although statistically significant, have modest odds ratios for CD but give useful clues to likely pathogenic mechanisms.

The genetic contribution to UC causation is weaker than for CD, though recent work has also questioned the importance of the genetic influence in CD course (60). In UC initially it was thought that only about one in ten identical twins of UC patients became affected compared with about one in two for CD, though more recent work shows even weaker concordance in UC. In CD location in MZ twins was concordant in 65% at diagnosis and 69% at ten years, whilst with UC MZ twins showed no concordance in disease extent at diagnosis or after 10 years. The figures for age at diagnosis for MZ twins with UC showed some concordance at 67 %, with symptomatic onset also concordant in 44 % (61). HLA-DRB1*0103 is strongly associated with extensive UC in caucasians, as is the multi drug resistance 1 (MDR1) gene (59). MDR1 expresses an apical epithelial efflux glycoprotein in distal small bowel and colonic epithelia. MDR1 gene-knockout mice have increased susceptibility to intestinal inflammation. TNF superfamily gene members have also been associated with IBD phenotypes, with both high and low risk haplotypes of the same gene (TNFSF15) identified.

These genetic studies are providing increasing evidence to implicate defects in phagocyte function in CD and defects in barrier function in UC. The implications of some of these gene associations for interaction with the intestinal microbiota is discussed in chapter two.

1.6 Environmental factors in the pathogenesis of inflammatory bowel disease

There are an abundance of hypotheses about the involvement of possible environmental factors in IBD and a number of risk factors have been identified:

1.6.1 The effect of diet

Diet can have both harmful and ameliorating effects in IBD, and this has been noted in both observational and interventional trials. A change in main dietary foodstuffs from rice to increased amounts of animal meat, fats and dairy products has been shown in a Japanese epidemiological study to precede increased incidence of IBD by approximately twenty years (62), other similar studies exist and a systematic review of the literature has demonstrated that high intake of total fats, omega-6 oils, polyunsaturated fatty acids and meat pre-disease are associated with an increased risk of both CD and UC. Obesity was associated independently with IBD disease activity (63), whilst high intake of fruit and vegetables was negatively associated with CD, with vegetables also negatively associated with risk of UC, but with omega-3 oils demonstrating no beneficial effect (63). The increasing incidence of IBD in areas previously thought to have low incidence like China, South Korea and Puerto Rico has been proposed to be due to the expansion of the 'western diet' (64). Diet can influence the microbiota colonising the intestines, and a comparison of fecal samples from European and African people, who eat a high fibre diet showed a preponderance of Bacteroidetes and low quantity of the Enterobacteraeciae and firmicutes seen in the European faecal samples (65). This dysbiosis has been associated with IBD (see

later), and it seems likely that diet plays a role in IBD, perhaps via this mechanism. CD patients have also been shown to have a higher than control or UC intake of sugar, though whether this is a primary or a secondary phenomena to CD is disputed (66;67) and intake of linoleic acid, an essential fatty acid found in many oils also seems to precede UC diagnosis (68). In areas of the world where soluble fibre, for example, in the form of plantains, are consumed in high quantity, such as the Caribbean and West Indies, incidence of CD is low and *in vitro* experiments have demonstrated an inhibitory effect of this plantain fibre on bacteria mucosal adhesion (69). The permitted food emulsifier polysorbate, a detergent contained in highly processed fatty foods, enhanced bacterial translocation through the bowel mucosa *in vitro*, and this suggests a mechanism by which highly processed food may worsen IBD or risk of developing IBD (69). Finally the importance of diet in IBD pathogenesis is confirmed by a meta-analysis demonstrating that specialised enteral feeds such as basic constituent (elemental) or polymeric diets, which have low residue and basic constituents excepting hydrolysed protein, can induce clinical remission in CD (70).

1.6.2 Hygiene

IBD has been reported to be commoner in people with reduced microbial exposure during childhood for a variety of reasons, the so called ‘hygiene hypothesis’, and factors important in this hypothesis may be *H.Pylori*, helminths, breast feeding and sibship (71). Most of these epidemiological studies are limited by methodology, with recall bias the most difficult problem. The hypothesis emerged when the observation was made that the incidence of IBD in developed and developing countries was rising

(72;73), and that this correlated with improved sanitation, hot water and less crowding and use of products like toothpaste. The basis of the hypothesis is that a reduced exposure to common infectious agents when young leads to a immune hypersensitivity response later on in life when challenged with the same bacteria that may be a component of, or cause the pathology seen in IBD (74). Larger family size is associated with lower rates of all atopic disorders, which may be due to differing levels of microbial antigen exposure in small and large families (75).

Improved hygiene is associated with decreased microbial exposure (76), and the development of immunological tolerance to these microbes, and the subsequent balance point between pro-inflammatory T-cells and regulatory T-cells may be impaired by this and be the cause of future hypersensitivity and diseases like IBD in patients (77).

A specific environmental microbe associated with reduced incidence, delayed presentation and a possible protective effect in IBD is *Helicobacter pylori* (78-80). In a study examining rates of relapse in IBD, amongst non-smokers, patients seropositive for *Helicobacter Pylori* were also less likely to relapse than in those seronegative for the bacteria, implying a possible protective role (81) which merits further evaluation.

With increasing urbanisation and modern hygiene practices there is also a lower exposure to soil borne helminths in infants, and helminths such as *Trichomona suis* induce a strong Th2 immune response, opposing the pro-inflammatory Th1 response (82;83) and providing an important role in regulation of the intestinal microbiota and their absence has been associated with the development of IBD (84). Chronic infection with helminths also induces production of the anti-inflammatory

cytokine TGF β which is a component of the regulatory T cell effect (85) and clinical trials with helminths in IBD have also shown some efficacy (86).

The ‘cold chain’ phenomenon is the observation that the introduction of the refrigerator is often related in time to CD outbreak (87). This is thought to be because some bacteria which can survive at fridge temperatures are also seen in CD lesions (88), namely *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium botulinum* and *Bacillus Cereus* (89). In some older IBD patients matched with controls, it was noted that the IBD sufferer owned a fridge 4 years earlier than the matched control patients (90). Whether other confounders which co-existed with the introduction of fridges such as increased electricity are more important is debated.

The importance of childhood infections below the age of 6 months in later development of CD has proved equivocal (91;92), but the impact of measles in particular has been debated. Measles infection has some biological plausibility, as it can colonise with some chronicity in the mesenteric endothelium and potentially cause the immunological changes seen in IBD (83), but no association has been found between measles in-utero and CD in a large study (93), and this discordance argues against causation.

Another more controversial factor that forms part of the hygiene hypothesis is whether childhood vaccination plays a role in subsequent development of IBD. Vaccination affects immune maturation, including that of the gut and could hypothetically play a role in IBD pathogenesis. One study suggested that those who had childhood vaccinations were 2.5 – 3 fold more likely to get UC or CD, but on closer scrutiny differential follow up terms were used between groups, which weakened the work (94), and other cohort studies did not support a pathogenic role for vaccinations in IBD (95),(96). A large case-control study was performed in

response to some public concern about the Measles, Mumps, and Rubella (MMR) vaccine and a link with IBD, and the results were negative (97).

Though antibiotics have been used to treat IBD in clinical trials and for perianal disease and abscesses in CD (see later), they have also been associated with risk of CD in some observational studies and though it has not been clarified which specific antibiotics are important, tetracyclines have some correlation with disease. Unfortunately studies were often confounded by other drugs which were co-administered and not accounted for which may have been more relevant (71;98).

Breast milk is a common environmental exposure for children which contains amongst other constituents immunoglobulins, and lactoferrin which has some antibiotic effect against particularly *Escherichia coli* (*E.coli*) and *Staphylococci*. Again some papers have cited a protective role for breast milk against future development of IBD (99;100) whilst others state that no difference was found (101).

Siblings may play a role in the development of IBD, with smaller family size associated with a decreased risk of IBD (102) and with an older sibling having an increased risk of developing UC with a younger sibling having a decreased risk of CD (103). The siblings of an older brother or sister are in general exposed to pathogens at an earlier age than they otherwise would be, though its not clear why the risks are in different directions in UC and CD, though immune repriming and repeated exposures to pathogens may have different effects in the different conditions.

The effect of living in an urban community versus a rural upbringing causing an increased incidence of IBD has its proponents (104), and opponents (105). The observation that attending day care centres in the first six months of life has an increased risk of adult CD also has positive and negative results (77;106), and living near a hot water tap in a household with a separate bathroom also has been observed

to be associated with a five fold increased risk of CD (107;108). Taken together, all these observations suggest that there is some plausibility to the hygiene hypothesis, and it may play some role in the greater than 50% environmental component responsible for IBD causation.

The studies involving the examination of the effect of siblings, breast milk, rural life and day care are all fundamentally limited by the effects of recall bias and prospective cohort studies are needed to examine these effects with more confidence.

1.6.3 Stress

Stress has been associated with IBD (43) and IBD has been reported to be higher in association with ‘white collar’ occupations, city living and higher income (109). ‘White collar’ occupations included instrument makers, electricians, sales representatives and office workers and their work may involve greater stress but interpretation may be confounded by higher physical exercise levels and open air exposure in the other jobs these were compared too, such as bricklayers and road construction workers. Stress can arise from external (exteroceptive) or internal (interoceptive) sources and intuitively for most patients and clinicians, stress would seem to worsen IBD. Though most longitudinal studies demonstrate that stress, adverse life events, or depression worsen the course of IBD, there is not yet good data to demonstrate that relaxation and stress reduction measures improve the clinical course (110). Mechanistically stress has also been associated with reactivation but not initiation of IBD in mouse models (111), and psychoneuroimmunological studies have evaluated the effects of the ‘brain gut’ axis comprising the hypothalamic, pituitary and adrenal tissue and the autonomic and enteric neuronal complexes, and

have provided a feasible biological mechanism by which psychological stress can affect mucosal inflammation. These include inducing low grade inflammation, affecting epithelial ultrastructure and consequent paracellular and transcellular permeabilities, increased bacterial translocation, and allowing access of the bacterial antigens to the lamina propria and antigen presenting cells (see later for the importance of these mechanisms) as well as affecting signalling by corticotrophin releasing factors (CRF) from mast cells and a reduction in the visceral sensitivity to nociceptive stimuli (110;112-114).

Psychotherapy has not been shown to alter IBD disease activity, even if it improves general psychological state, and so cannot be generally advocated for all chronic IBD patients, though the therapy probably works best for those who have psychological problems related to their IBD (115). More recent studies showed reduced relapse rates in those given psychological counselling, and reduced outpatient attendance compared to the pre-counselling year (116). In addition CD patients who use 'unfavourable' coping strategies least have lowest rates of relapse after one year follow up (117). The role of stress modification strategies such as hypnotherapy and antidepressants in treatment of IBD also needs further delineation, and improvement of support networks, be they family based or the resources of the national IBD patient associations is intuitively related to better quality of life, with absence of these associated with worse outcomes (110;118).

1.6.4 Smoking

Smoking both increases the risk of developing CD, and worsens the course of the disease, with smokers experiencing increased relapse and surgical rates (119).

Conversely smoking has been associated with decreased UC risk with ex-smokers being at increased risk of relapse (63;120). In CD smoking has been associated with increased steroid and immunosuppressant requirement, and increased rate of reoperation (68) with increased ileal and decreased colonic incidence in most studies (121;122). It has correspondingly been associated with increased rates of penetrating disease (123).

1.6.5 Microbiota and surgical factors in IBD pathogenesis

Infectious agents may have a key role in IBD, and have been investigated extensively, particularly *Mycobacterium avium paratuberculosis*, or MAP, (124) and *E. coli* (125), and these bacteria amongst others are discussed in some detail in chapter two.

Prior tonsillectomy and appendicectomy (126) appear to increase risk of CD, but appendicectomy reduces the risk of UC.

1.7 Treatment of IBD

For many years the treatment for both conditions has been corticosteroids for acute attacks and oral 5-aminosalicylic acid (5-ASA) compounds for maintenance, with immunomodulation with azathioprine or mercaptopurine added for patients with chronic relapsing disease. Newer biological agents such as the tumour necrosis factor α (TNF α) binding antibody infliximab have helped substantially (127) Recently there have been meta-analyses for most therapies used in IBD (128).

1.7.1 Anti-inflammatories - 5 ASA compounds

5-ASA compounds contain a salicylate moiety, and they improve disease course in both rheumatoid arthritis, where their effects were first noticed, and IBD (128). Sulphasalazine induces dose related reduction in male fertility and rarely blood dyscrasia (129). It is the mesalamine component of 5-ASA compounds that accounts for their efficacy in IBD and different delivery formulations exist (128;130) with enema preparations also showing clinical efficacy and serious side effects are rare (131). Meta-analysis of 11 randomised controlled trials (RCT) confirms the ability of 5-ASA compounds to both induce remission in active UC and prevent relapse in quiescent UC, with an optimum dose of 2.0 to 2.4g for induction of remission and relapse prevention with no greater response seen for induction of remission in UC with higher doses than 2.4g, though trials comparing relapse prevention in UC with higher doses were small in number (132). A previous Cochrane Database review examining the effect of the 5-ASA compounds use for treating CD was more

equivocal about the efficacy of 5-ASA drugs in CD (133), and this was supported by more recent meta-analyses of the effect of 5-ASA compounds in inducing remission in CD relapse – a very modest but statistically significant clinical effect was seen (128;134). 5-ASA drugs were not recommended as only one trial was deemed to have low bias (135). In terms of preventing relapse of quiescent CD there is also poor evidence (136;137).

1.7.2 Steroids

Steroids are used in a variety of conditions including respiratory and rheumatological problems and the first RCT evaluating the effect of cortisone in UC with placebo was performed in 1954 (138). Steroids have a multitude of effects on the immune system, including reducing immune cell migration to a site of damage, reducing cell adhesion molecule expression, inhibition of neutrophil migration to an area of damage and decreasing pro-inflammatory cytokine production as well as inducing apoptosis of lymphocytes. Most of these effects are mediated by increased transcription subsequent to agonist binding glucocorticoid receptors in the cell nucleus (139;140). They have significant side effects which include an increased risk of infection (141), reduction in bone density with prolonged courses of treatment, and Diabetes Mellitus (142), though budesonide (143) and beclomethasone have significantly fewer side effects. The effects of steroids on macrophage function are evaluated in this thesis in chapter 8.

A meta-analysis of five trials demonstrated that steroids were effective in inducing remission in relapses of UC (144), with a single trial demonstrating efficacy of intravenous (IV) hydrocortisone (145), though quality of evidence overall was low

as only 445 patients were included (128). Two RCT indicated that steroids could also induce remission in CD relapse (135;146) with standard steroids inducing remission in 60 % of patients compared to the 31 % observed with placebo. There was no evidence for IV hydrocortisone use compared with oral steroid in CD relapse, and the oral steroid trials involved a relatively low total of 247 patients too, but statistically significant effects were seen in both trials. Similarly two trials of the efficacy of budesonide in CD relapse showed efficacy (147;148) in a small number of patients (458, with terminal ileal or right sided CD), with studies comparing standard steroids and budesonide showing a modest superiority of standard steroids over budesonide at the cost of an increase in side effects attributable to the standard steroids (144). Five trials assessed efficacy of budesonide in prevention of relapse in quiescent CD with no benefit seen, though it obviously can be considered to limit side effects in steroid dependent disease (128).

1.7.3 Immunosuppression

The immunosuppressants 6-mercaptopurine (6-MP) and its precursor azathioprine are used as maintenance therapy in IBD. Other drugs used as immunosuppressants are methotrexate, cyclosporin, and tacrolimus. Following success in rheumatological disease, initial reports of the utility of azathioprine and 6-MP (149;150), prompted further clinical trials of all these immunosuppressants, though weaknesses in study design and analysis have prompted a recent meta-analysis to clarify their most appropriate use in relapse and remission in IBD (151). Each drug has separate mechanisms and some of the potential subtleties of these are discussed in chapter eight. Side effects from this group of drugs can be significant and require monitoring

(152;153). They include nausea, infection, allergy, bone marrow suppression, acute pancreatitis, hepatitis, and cancer (azathioprine and 6-MP), with methotrexate potentially inducing hepatitis, pneumonitis, infection, malignancy, myelosuppression, alopecia and stomatitis. The main concern in the use of cyclosporin and tacrolimus is renal toxicity, which is closely monitored, but they can also be associated with neurological symptoms (152).

Both azathioprine and 6-MP were not found to be significantly useful for induction of remission in UC (154;155) with methotrexate similarly ineffective (156), but with all three drugs assessed on minimal RCT information (128). Only one RCT of cyclosporin was evaluated and this involved twenty patients treated with either 4mg/kg IV cyclosporin for seven days followed by oral therapy versus placebo in patients admitted with steroid refractory acute UC, and 'response' was significantly greater in the cyclosporin group, with response defined as an improvement in a non standard clinical response score ranging from 0 – 21, for no symptoms ranging to severe symptoms (157). Two other clinical trials lacked a placebo arm and were not included in analysis (128) (158;159). Tacrolimus was also ineffective at inducing full remission in patients with mild to moderate UC (160), though some response was seen.

The meta-analysis (151) of the effect of azathioprine for maintaining remission in UC demonstrated benefit, based on annual relapse rates of 39% and 66% in the azathioprine and placebo groups respectively, with 3 RCT included and suggesting that the best time to instigate azathioprine treatment is during remission, rather than during relapse, and increased relapse rates were seen in the year following withdrawal of azathioprine therapy (161). Trials of methotrexate also found the drug equally unhelpful in maintenance of remission, certainly in oral form (151;162).

In CD, azathioprine, 6-MP (156), and cyclosporin (163) were all ineffective for the induction of remission (151), but intra-muscular (I/M) methotrexate proved to be effective, even if only when co-prescribed with steroids at levels > 20mg/day (164). Tacrolimus proved of some utility in treating fistulae (165).

In the analysis of immunosuppressants (151) which could prevent relapse azathioprine (166;167), 6-MP and methotrexate were all considered capable of maintenance of remission with relapse rates higher when azathioprine was withdrawn (151) and the beneficial steroid sparing effects of azathioprine in corticosteroid dependent patients were also noted (168). Finally both azathioprine, and 6-MP (169) were better at preventing post-operative recurrence of CD. Methotrexate had a significant effect at preventing relapse in CD patients (170), but cyclosporin was as ineffective for maintaining remission as it was for preventing relapse (128).

1.7.4 Biological Therapies

Anti TNF α therapies target the dominant common inflammatory cytokine signalling pathway in IBD to prevent mucosal inflammation. Adalimumab (Humira®) is an anti-TNF-alpha recombinant human IgG1 monoclonal antibody (171), whilst certolizumab pegol (Cimzia ®) is a pegylated TNF α blocker – a recombinant humanised antibody fragment against TNF α (172). Infliximab (Remicaide®) is a chimeric anti-TNF-alpha monoclonal antibody (173). Other treatments exist to modulate TNF signalling such as etanercept and thalidomide but are not in widespread use and not discussed here.

Antibody therapies against integrins such as the anti $\alpha 4$ integrins (natalizumab) block the adhesion and migration of leukocytes into the gut, and as this

has been associated with CD pathogenesis are potential therapeutic targets (174). RCT have been conducted in this group of drugs and their utility is evaluated too.

Anti TNF α antibodies had initial impressive results in rheumatoid arthritis (175), and subsequently were tried in IBD, and now form an important part of recent American and European guidelines for the treatment of CD and UC (128;176;177). A meta-analysis of all current data on biological therapies which examines double blind RCT has been performed to assess both their efficacy and safety (178). The chief risks from these therapies are opportunistic infection and lymphoma (142;179), with risks increased when these drugs are used in combination with immunosuppressives. Congestive heart failure, demyelinating disease, lupus like syndromes and induction of autoantibodies are other potential sequelae (180).

Infliximab (IFX) is effective at inducing remission in moderate to severely active UC (181;182), as well as in improvement of the condition of hospitalised patients with severe disease, (183;184) though the recommendation was given without statistically significant results in clinical response. There were insufficient data to comment on the utility of IFX as a maintenance therapy for UC (128), as two trials evaluating this only really examined prolonged induction (182).

The efficacy of IFX, adalimumab (ADA), and certolizimab pegol (CTZ) for induction of remission in CD was evaluated in patients with difficult disease at 4 -12 weeks, producing remission in 28% of patients in the treatment arm, compared to 19% on placebo. These differences are smaller than those seen with other treatments analysed above, but represent a difficult 'refractory' group of patients (128;185-187).

Anti $\alpha 4$ integrins are effective at inducing remission (188;189) in CD patients, but have the serious risk of progressive multifocal leukoencephalopathy (190;191), and if used are reserved for patients refractory to anti – TNF α therapy.

In a meta-analysis of 1390 patients the anti – TNF α therapies were found to be effective at prevention of relapse in CD (192-194). Relapse occurred in 56% of patients on IFX, ADA or CTZ therapy and in 78% of patients on placebo treatment. Further benefit is likely when anti – TNF α therapy is combined with immunosuppressives like azathioprine, though this benefit has so far been seen with IFX and azathioprine together (195), but not with methotrexate. Natalizumab in one RCT has also been demonstrated to prevent relapse in CD, but is used as second choice after anti – TNF α therapy (188).

1.7.5 Antibiotics

Immunosuppressant and biological therapies are clearly effective, and may modulate the gut immune response to triggering antigens or bacteria, but altering the gut microbiome using probiotics is in its infancy with little efficacy seen in either UC or CD with their use (196;197). Antibiotic use generally targets bacteria hypothesised to be causative or involved in CD such as *Mycobacterium paratuberculosis*, *Listeria*, or *Escherichia coli* (87;124;198-200). Antibiotics and meta-analyses of their efficacy are discussed in detail in chapter two, section 2.9.

1.8 Implications of IBD gene-associations for interactions with the gut microbiota – evidence for disordered innate immunity and mucosal barrier function

Sections 1.5 and 1.5.1 explain the familial clustering seen in IBD as well as the gene defects identified so far. How some of these defects might affect mucosal barrier function and interaction with the microbiota is explained below.

1.8.1 NOD2/CARD15

The NOD2/CARD15 protein is expressed intracellularly in macrophages, epithelial cells and Paneth cells. The CD-related NOD2 mutations all affect the leucine rich region (LRR) which acts as the receptor for the bacterial cell wall proteoglycan component muramyl dipeptide (MDP). There has been uncertainty about whether these are loss-of-function or gain-of-function mutations, but the consensus is moving towards regarding them as loss of function.

The NOD2 mutations produce different impairments in bacterial handling depending on the cell type involved. Defensin levels are noted to be low in the Paneth cells (present at the base of small intestinal crypts and some colonic crypts, particularly proximally) of individuals with mutant NOD2 (47). However recent work has shown defensin deficiency to be independent of NOD2 status, and perhaps secondary to mucosal inflammation (201), so this remains controversial. NOD2 mutant macrophages have diminished IL-8 response to MDP (47) and this reduced IL-8 response correlates with reduced neutrophil recruitment and generally defective acute inflammatory processing of bacteria in CD (202).

NOD2 -mutant mice are particularly susceptible to challenge with oral *Listeria monocytogenes* (47), have decreased β defensin secretion and increased nuclear factor kappa B (NF κ B) pro-inflammatory signalling in response to MDP(47). It seems possible that a point is reached in IBD where NF κ B signalling becomes pathological rather than homeostatic, and the NOD2 mutation could lower the threshold at which this point is reached.

There is considerable cross-talk between NOD and TLR signalling with Myeloid differentiation factor 88 (MyD88) as an essential core intermediary (203) (Figure 1.2).

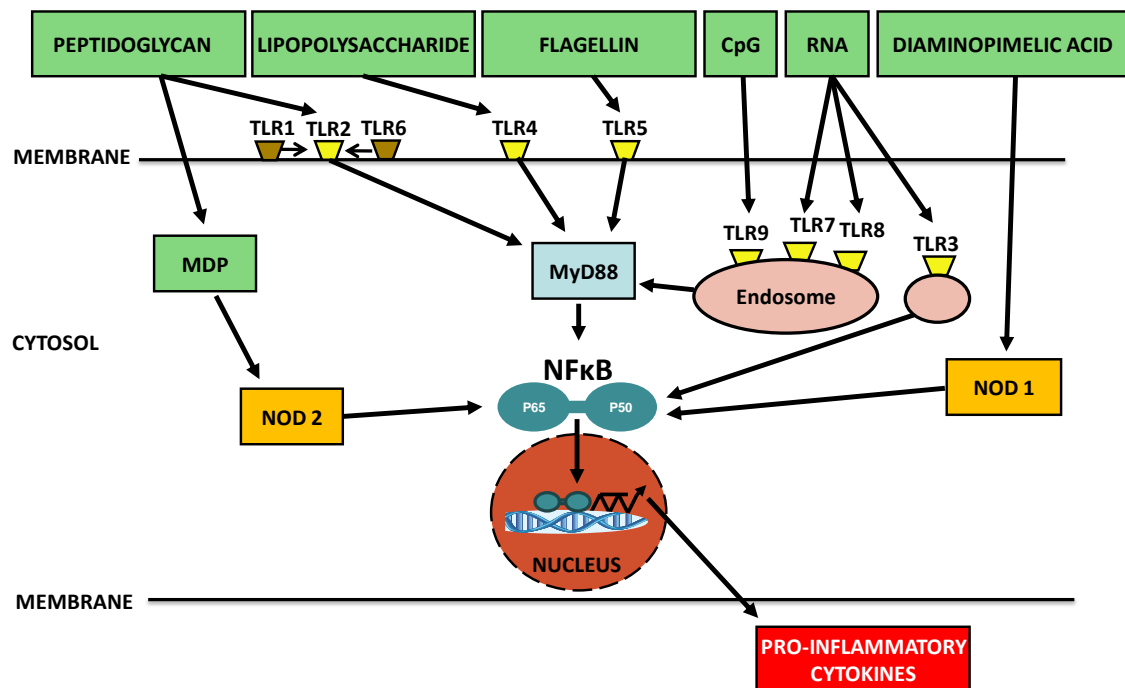


Figure 1.2 (from (125)) NOD/TLR signalling in a mammalian host cell. Sensing of molecular moieties produced by invading microbial pathogens (so-called pathogen-associated molecular patterns or PAMPs) occurs both on the cell-surface and within the host cytoplasm, mediated by PRRs. These include TLRs triggered by bacterial moieties. MyD88 is an essential core intermediary for most TLRs leading to activation (increased nuclear localization) of transcription factor NF κ B the key cell signalling molecule for inflammation. Of note, TLR5 is sited basolaterally and stimulated by bacterial flagellin. Nod-like receptors also activate NF κ B via recruitment of RIP2 kinase, triggered by peptidoglycan moieties present within Gram-negative and Gram-positive bacteria cell wall, e.g. L-Ala- γ -D-Glu-*meso*-diaminopimelic acid (triDAP) and MDP MurNAc-L-Ala-D-isoGln. There is possible cross-talk between NODs and TLR signalling pathways (203).

1.8.2 The role of Defensins in innate immunity (204)

The strong expression of the Crohn's-associated gene NOD2/CARD15 in Paneth cells, the main source of bactericidal defensins, has led to great interest in the role of defective defensin production/function in CD pathogenesis. Paneth cells are particularly plentiful in the distal small intestine and the typically low number of bacteria in the small intestine may be partly due to the high abundance of α -defensins (cryptidins). Defensins can also act as a chemoattractant for cells expressing chemokine receptor CCR6, such as dendritic cells (a specialized antigen-presenting cell) and help to integrate host innate and adaptive immune response (204).

There is increased mucosal expression of human β -defensin 2 in UC, which may reflect the type of epithelial interaction involved in the pathogenesis. Mucosal peptides extracted from patients with CD, UC and healthy individuals showed different abilities to kill bacteria, as assessed by flow cytometry. There is a decreased anti-microbial effect in CD, when compared with UC, independent of inflammation status or concurrent steroid treatment, and notably a decreased anti-microbial effect on *Escherichia coli* (*E. coli*) – a key pathogenic suspect (204). In ileal CD, there is reduced expression of 'wingless-type' (Wnt) signalling, a regulatory pathway directing cell–cell interactions and transcription factors. This leads to reduced Paneth cell differentiation and reduced defensin release and might be a primary problem in ileal CD (204).

1.8.3 Defective phagocyte clearance

There are marked similarities between CD and the intestinal lesions found in patients with inherited phagocyte disorders such as chronic granulomatous disease and glycogen storage disease type 1b in humans. Further support for the defective phagocyte hypothesis comes from the identification of autophagy gene mutations in association with CD.

There is also good evidence that macrophages in CD tissue contain bacteria. Immunocytochemical searches for bacterial antigens in mesenteric and lymph node specimens from patients with CD show that macrophages and giant cells contain *Listeria* spp., *E. coli* and Streptococcal antigens. These were present near ulcers, fissures, abscesses and in granulomata, as well as within germinal centres of mesenteric lymph nodes (88). *E. coli* DNA has also been identified by laser capture dissection of CD tissue granulomas (205), and was found in 12/15 CD granulomas compared with 1/10 from Controls.

An adherent invasive *E. coli* (AIEC), LF82, isolated from a patient with ileal CD, has been shown to be endocytosed normally by macrophage endomembrane organelles and then to replicate within mature phagolysosomes without triggering host cell lysis (206). There is also evidence that AIEC induce macrophages to form granulomata (207).

Bacteria can also suppress macrophage responses. Previous work by our group has shown that a mannose-containing glycoconjugate that is expressed by *Mycobacterium paratuberculosis* as well as by *Candida albicans* and other yeasts (but not *E. coli*), is

able to inhibit the killing of *E. coli* by macrophages (208). This effect is dependent on the MyD88 regulatory pathway and TLR4. A circulating anti-mannan antibody is commonly found in CD, the anti-*Saccharomyces cerevisiae* antibody (ASCA). Thus, the pathogenesis of CD may be contributed to by primarily impaired phagocyte function, secondary suppression of macrophage function by bacteria, or both. Bacteria and their interaction with macrophages are discussed in more detail in chapter two

1.8.4 Neutrophils

The neutrophil is the ‘professional killer’ of bacteria and it has been suggested that defective neutrophil recruitment as a consequence of reduced IL-8 expression by mononuclear cells, e.g. as a result of NOD2/CARD15 mutation, may result in macrophages being left to clear bacteria which they are less competent to kill (209). Repeat mucosal biopsy, hours apart, at the same site of the rectum in CD, UC and Control patients, resulted in lower expression of IL-8 and fewer neutrophils being recruited into the biopsy site in CD intestinal tissue (202). Neutrophil migration into skin blisters in CD patients was also reduced (210). This reduced migration of neutrophils exposes further the already ‘autophagy impaired’ macrophages ability to clear bacteria.

1.8.5 Mucosal barrier

The integrity of the mucosal barrier in the intestine relies upon the mucus layer (and associated secreted proteins), the underlying glycocalyx and the epithelial cell membranes and tight junctions. The mucus layer is continuous in the colon but discontinuous in the small intestine. The mucosal barrier has been shown to be compromised in IBD, and in unaffected relatives of CD patients (211) where it may precede lesion development (212).

The mucosal barrier weakness may be genetic or acquired. Absence of the apical efflux glycoprotein gene MDR1, and also possibly abnormalities of the trans-membrane mucin MUC3 are associated with UC (59). Acquired barrier defects can also occur, although may be at least in part secondary to inflammation. *In vitro* studies have demonstrated that pro-inflammatory cytokines such as interferon-gamma and TNF alpha, which are increased in the intestinal mucosa of patients with IBD as a result of bacterial NOD/TLR stimulation or through hypersensitivity, can induce mucosal permeability, mediated by internalization of junctional proteins (212).

The activation of NF kappa B in epithelial cells is an early event in IBD that can be found in the surface epithelium of unaffected identical twins of IBD patients, even in the absence of histological inflammation. This seems likely to be a consequence of bacterial–epithelial interaction and may again reflect a barrier defect (213). Weakness of the mucosal barrier allowing flagellin to pass through tight epithelial junctions and thus have access to basolateral TLR5 with subsequent triggering of IL-8 release has been described in mouse models (214), and represents a plausible model for UC, perhaps with inflammation perpetuated by autoimmunity (215).

The above defects in innate immunity and mucosa barrier all support the hypothesis that CD results from an altered response to the gut microbiota which is now discussed.

CHAPTER TWO : THE ROLE OF THE INTESTINAL MICROBIOTA IN THE PATHOGENESIS OF INFLAMMATORY BOWEL DISEASE

2.1 Diversity in the Gastrointestinal tract

The human gastrointestinal tract is colonised by 500-1000 bacterial species with 80% of these yet to be cultured (216). In the average individual metagenomic sequencing studies have demonstrated that at least 160 species are harboured, and largely shared (217). The bacterial population increases distally along the small bowel with gram negative aerobes and some obligate anaerobes present, whilst the colon is heavily populated with anaerobes, approximately 10^{14} per gram of luminal content (47). The use of culture-independent techniques such as real time polymerase chain reaction (PCR) (218), FISH (127;219), ribosomal intergenic spacer analysis (RISA)(220;221) and 16S ribosomal DNA (rDNA) (218;221) sequencing can allow analysis of microbes involved in CD by their expressed genes and demonstrate their cellular location. It can provide additional insight into the microbiota changes in CD, particularly when a combination of techniques is used (218).

2.1.1 Loss of diversity in IBD

Automated ribosomal intergenic spacer analysis (ARISA), terminal restriction fragment length polymorphisms (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) have shown reduced diversity in both faeces and mucosa-associated microbiota in IBD (222;223). In one study, species richness increased from control to non inflamed UC and CD tissue but then declined in more inflamed tissue (223). *Firmicutes* and *Bacteroidetes* diversity are particularly affected, though ARISA demonstrated an increase in diversity of less well characterised *Firmicutes*.

2.2 Alterations in the faecal microbiota in IBD

There is clear evidence that up to 50% of relapses of UC and CD may be triggered by infection with conventional pathogens such as *Salmonella* spp and *Campylobacter* spp (47;224;225) or related to foreign travel. Generally though, the faecal microbiota in IBD lack any obvious conventional pathogens. The faecal microbiota do not reflect the mucosa-associated bacteria, which may be of much greater significance in IBD. FISH probes can be used to identify 11 bacterial groups in healthy patients and those with IBD and demonstrate distinct differences between faecal samples (219). Depletion of *Faecalibacterium prausnitzii* (*F. prau*) bacteria in CD, and conversely normal *F. prau* in UC enabled recognition of active UC or CD with good sensitivity and specificity. More recent twin studies which examined biopsies from multiple sections of the intestinal tract within an individual from ileum to rectum, showed that *F. prau* was consistently present (for colonic CD or healthy controls), or consistently absent throughout (for ileal CD) (226). In other work there

was also no association found between depletion of *F. prau.* and isolated colonic CD, though this was seen in ileal CD (227). In animal models there is a probiotic effect demonstrated by the supernatant of *F. prau.* (228) and also a negative association between *F. prau.* and recurrence of ileal CD after surgery (228;229). Differences in bacteria numbers and species in the colonic bacteria in the mucus layer have also been noted (219). Other changes in IBD faecal microbiota included depletion of *Clostridium coccoides* in UC and *Clostridium leptum* in CD (47).

Bacteroides fragilis has been shown to account for 60% of the biofilm (mucosa associated bacteria within the mucus layer) mass of IBD patients and *Eubacterium rectale- C. coccoides* in 15% of the same biofilm (47). Culture-independent analysis of ileal mucosa has also demonstrated relative depletion of *Clostridiales* but an increase in invasive *E. coli* in ileal CD (218). *Listeria* and *streptococcal* antigens have also been found in CD tissue (88) , but further attempts to identify *Listeria* by PCR have been negative (47).

Emerging evidence also suggests that genetics and/or environmental exposure during childhood, in part, may determine the gut microbial composition. Using T-RFLP molecular fingerprinting, (230) recently demonstrated not only that that the faecal microbiota was more similar between healthy identical twins than between twins with CD, but that there were significant differences between identical twins concordant and discordant for CD.

2.3 Growing evidence for a pathogenic role of *E. coli* in CD

The colon, unlike the small intestine, has a near continuous mucus coat, and the bacteria adherent to this may differ in type (eg anaerobic) and number to those under the mucus -a microaerophilic niche (231). Aerobic culture of colonoscopic biopsies after removal of mucus is often sterile in control colons, but in CD contains increased bacterial numbers. More than half of these are *E. coli* (232), even though they represent less than 1% of the faecal microbiota. A similar result has also been seen in ileal mucosa in CD (233). Adherent and invasive *E. coli* (AIEC) have been found in 21.7% of CD cases versus 6.2% in controls. At least eight groups have identified independently an increase in mucosa-associated *E. coli* in CD (215), and culture-independent molecular techniques have confirmed this (218;220). Two of these studies also demonstrated increased numbers of *E. coli* in UC.

E. coli DNA has also been identified by laser dissection of CD tissue granulomas (205), and was found in 12/15 CD granulomas compared with 1/10 from 'controls'. RISA has been used to identify DNA segments more commonly present in CD and UC mucosal biopsies than controls (220). Five segments specific for CD mucosa have been sequenced and all found to contain *E. coli* DNA. Poor correlation was noted between site of inflammation and *E. coli*, compatible with *E. coli* being a causative organism rather than simply colonising inflamed mucosa. The *E. coli* isolates identified by RISA were more likely to be from phylogenetic groups B2 and D (220), characteristically more likely to be pathogenic at extraintestinal sites and adherent to intestinal cells. Group B2 *E. coli* have virulence factors conferring strong colonising capacity (234), and this provides an explanation for their increased prevalence in CD mucosa and also for the tendency for abscess and fistula formation

in CD. *E. coli* isolated from ileal biopsies in patients with CD have been shown to be adherent and invasive in epithelial cell lines, and able to survive and replicate within macrophages (206) without causing cell death but inducing secretion of the proinflammatory cytokine tissue necrosis factor α (TNF α). A broad spectrum of faecal microbiota lacking these virulence factors may invade after a mucosal breach.

2.3.1 Genetic basis for the A, B, and D phylogenetic classification of *E. coli*

Phylogenetic analysis has shown that *E. coli* has four main groups, A, B1, B2 and D. These groups can be classified by multilocus enzyme electrophoresis or by ribotyping, but it is more expedient to perform a triplex PCR using the genes *chuA* (haem transport), *yjaA* (unknown function from K-12 bacteria), and an anonymous DNA fragment TSPE4.C2 (235) which gives excellent correlation with standard methods. Previous work has shown that phylogenetic groups B2 and to a lesser extent D contain virulent extra-intestinal strains (236-238).

2.3.2 *E. coli* virulence factors

CD *E. coli* do not possess any known invasive determinants seen in other groups of *E. coli* but interact with epithelial cells via type 1 pili, flagellae, outer membrane vesicles, and outer membrane protein C (239). Mucinases are also produced by CD *E. coli* which more commonly possess serine protease autotransporters (SPATES)(220), perhaps explaining why they have been found within and beneath the mucus layer. Bacteria in the B2 phylogenetic group characteristic of CD patients (220) have enhanced persistence in the colon which is

dependent on P fimbriae (facilitating adherence) and aerobactin, an iron trapping compound (234). Group B2 seem to have accumulated virulence factor genes and it can be speculated that improved hygiene has possibly reduced expression of other *E. coli* groups in the West (234).

Type 1 pili of *E. coli* mediate bacterial adherence to ileal epithelia, and mannose has been shown to diminish this adherence (240). The Carcino-embryonic antigen-associated cell-adhesion molecule (CEACAM) 5 (otherwise known as CEA) and CEACAM 6 glycoproteins have been shown to be overexpressed in ileal epithelial cells in CD and *E. coli* adherence is prevented in vitro by blockade of CEACAM 6 (240). *E. coli* induces expression of CEACAM 6 on cultured intestinal cells directly, and also indirectly by induction of macrophages to secrete TNF α which increases CEACAM 6 levels (240).

Receptor binding events can also now be analysed at the level of a single bacterial species via flow cytometry. CEACAM receptors can be solubilised as probes to identify CEACAM binding of single bacteria, which can be decorated with fluorescent markers. This advance may be used to explore host-bacterial interactions in future experiments (241).

2.4 M cells as portals of entry

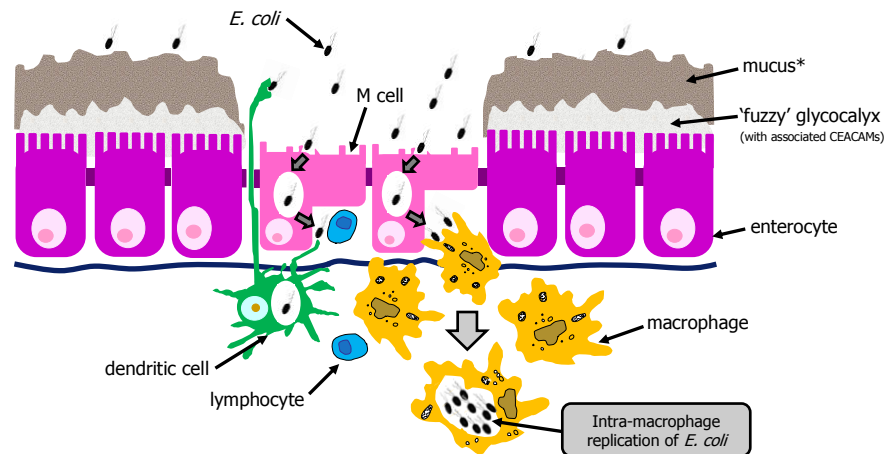


Figure 2.1 M cell interactions in CD NB – not to scale: the mucus layer >100 μm , is considerably thicker than the glycocalyx at <1 μm . (figure from reference (125))

An illustration of how adherent and invasive *E. coli* may gain access to the mucosa via M (microfold) cells in CD mucosa is shown in figure 2.1. It seems likely that AIEC found in CD, although lacking conventional pathogenicity genes, will also have to enter via specialized M cells within the FAE overlying Peyer's patches and isolated lymphoid follicles, and not through ordinary surface epithelial cells. M cells account for approximately 5% of the epithelial cells in the dome epithelium that overlies Peyer's patches and lymphoid follicles. They arise as a result of differentiation from surface epithelial cells stimulated by as yet unknown factors

released by the underlying B lymphocytes. Antigen sampling by these M cells is thought to be an early step in the development of mucosal immunity. The lack of mucus and the 'fuzzy' glycocalyx (which has the associated CEACAMs) facilitates contact between bacteria and the M cell-surface, followed by microbial antigen presentation to lymphoid or antigen-processing cells in the sub-epithelial tissue. AIEC are shown replicating within macrophage vacuoles (242). Bacteria are also translocated from the lumen by dendritic cell processes extending between epithelial cells (243).

2.5 Diarrhoeagenic *E. coli* epithelial interactions

As well as the AIEC seen in CD there are a number of different pathotypes of *E. coli* whose pathogenicity and method of epithelial interaction varies according to their virulence factors including EPEC (entero-pathogenic *E. coli*), and ETEC (entero-toxigenic *E. coli*). These bacteria interact with gastro-intestinal epithelia to colonise and invade tissue and cause pathology in diverse ways and to different degrees. Diarrhoeal illness is a common cause of illness and death worldwide, particularly in developing countries (244), and though worldwide incidence is falling, infantile diarrhoea cases are rising. (245) The various pathotypes of *E. coli* mean that different subtypes can induce diarrhoea without conferring group protection against other diarrhoeagenic *E. coli* pathotypes (DEP). In studies that have looked at a broad range of aetiological organisms causing diarrhoea, *E. coli* are pre-dominant. In some series *E. coli* was detected in 20% of cases where a bacterial cause was suspected (244;246).

Research has shown five types of DEP unequivocally associated with diarrhoea, distinguishable from non pathogens by virulence genes. There are global variations in monitoring and epidemiology of the pathotypes but they represent a high disease burden, particularly in areas like sub-Saharan Africa. (244;247). Experiments *in vitro* with diarrhoeagenic *E. coli* are discussed in chapter nine.

2.5.1 EPEC types and mode of epithelial interaction

Bray identified an EPEC, 0111, in the 1940's as a probable cause of infantile diarrhoea (*Bacterium coli* var neapolitanum) (244) and *E. coli* have been recognised as DEPs since then and the role of EPEC in infantile diarrhoea was verified by volunteer experiments (248).

EPEC can be divided into 'typical' and 'atypical' types. They are distinguished from entero-haemorrhagic *E. coli* (EHEC) by absence of the phage borne shiga toxins (244). Typical EPEC strains carry genes for the LEE (locus for enterocyte effacement) on a chromosomal pathogenicity island and virulence plasmids bearing genes encoding bfp (bundle-forming pili) and EAF (EPEC-adherence factor) whilst atypical EPEC do not have the EAF-plasmid or EAF-associated virulence plasmid. (244;249). The LEE factors give the EPEC its unique interaction with epithelial tissue as shown in figs 2.2 and 2.3. and allows serotype independent identification of EPEC (250). EPEC initially adheres to host epithelial cells utilising its bfp virulence factors, which co-ordinate bacterial aggregation (249). Subsequently, EPEC secretes several virulence factors by an LEE encoded type III-secretion system used also by other

organisms. The type III secretion system delivers proteins which promote diarrhoeal illness (251) but the bacterium adheres tightly to the host by secreting its Tir (translocated intimin receptor) receptor into the intestinal cell, and binding to this with its outer membrane ligand, intimin, another LEE factor, which can also bind β 1-integrins and invoke cytoskeletal re-arrangement. Actin, -actinin, talin, and ezrin proteins are recruited to the site of EPEC attachment following Tir-intimin binding, resulting in a unique pedestal-like structure where the pathogen resides, and this binding also causes microvillous effacement. This re-organisation is called an adherent-effacement lesion (A/E, see figure 2.2)(249;252). When the Tir is intimin bound it is tyrosine phosphorylated initiating signal transduction involving activation of phospholipase C (PLC), protein kinase C (PKC), inositol triphosphate (IP_3) flow, and intracellular Ca^{2+} release ultimately producing the cytoskeletal re-arrangement seen (figure 2.3)(253).

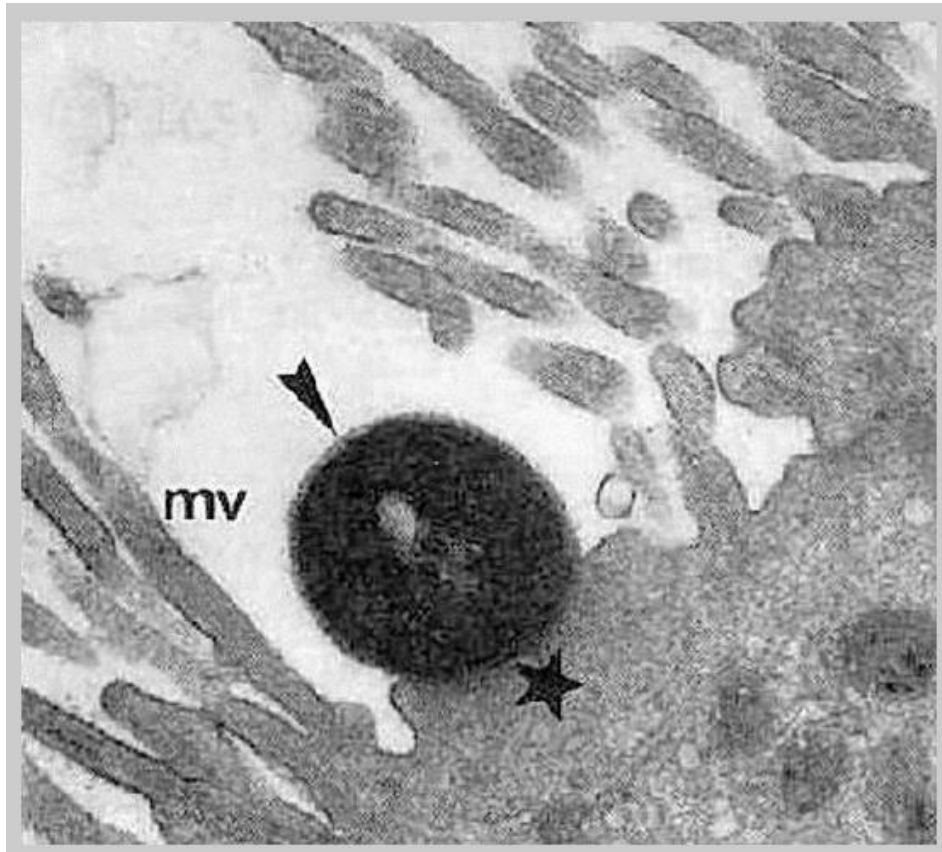


Figure 2.2 Transmission electron micrograph of an A/E lesion formed by EPEC infection of intestinal epithelial cells. From (252), Trabulsi *et al.* mv = microvillous effacement. Star demonstrates pedestal formation.

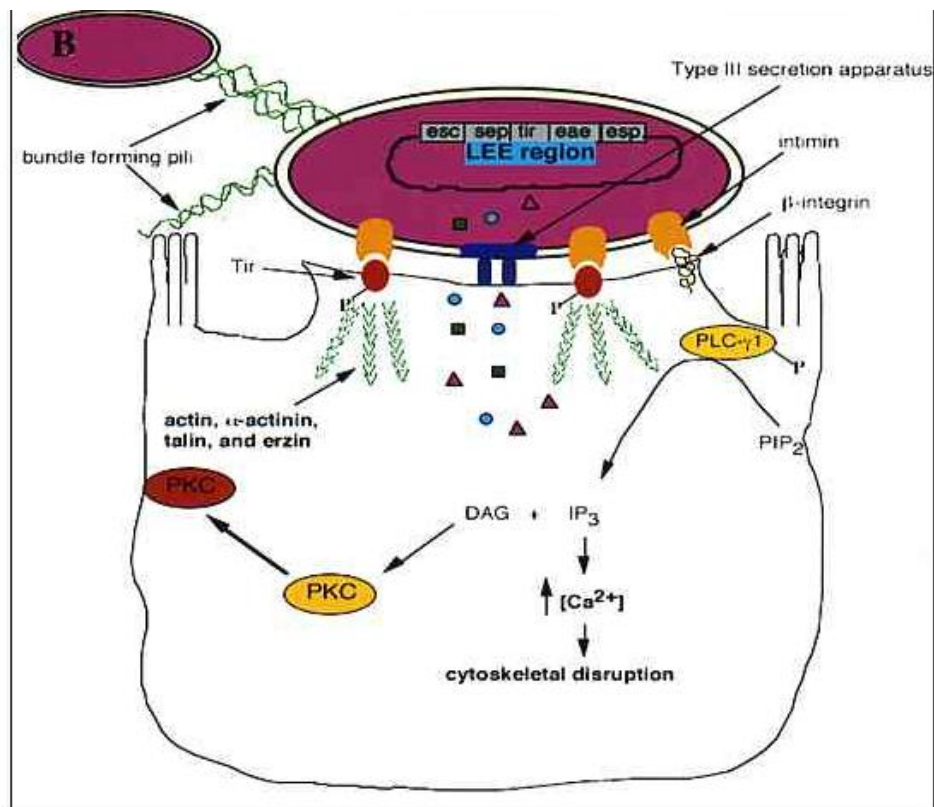


Figure 2.3 Signal transduction following EPEC Tir –intimin binding, from (249).

2.5.2 ETEC toxins and interaction with intestinal epithelium

ETEC are amongst the most common causes of travellers diarrhoea (244;254), and also a common cause of infantile diarrhoea, spread by contaminated food and water sources (255). Following adhesion to intestinal mucosa by fimbriae, they utilise a heat labile (LT) and heat stable (ST) toxin which causes diarrhoea. LT toxin is very similar to the *cholera* toxin which ADP-ribosylates G proteins (256), and antibodies against the *cholera* toxin can provide some treatment for travellers diarrhoea caused by ETEC. The ST toxin has no homology to cholera toxin (257). Disease is caused by

toxins rather than mucosal invasion, though inhibition of bacterial proximity to the intestinal mucosa intuitively seems likely to lessen damage.

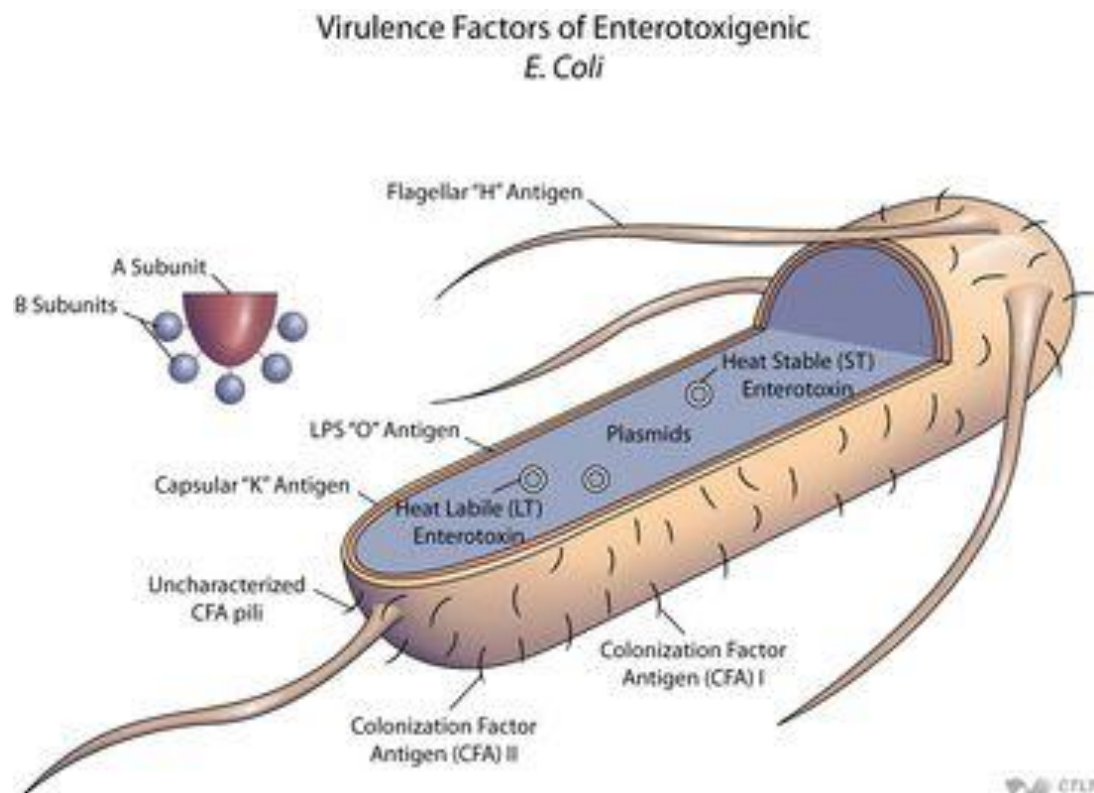


Fig from – "Virulence factors of enterotoxigenic *E. coli*" from Epidemiology of Infectious Diseases. Available at: <http://ocw.jhsph.edu>. Copyright © Johns Hopkins Bloomberg School of Public Health. Creative Commons BY-NC-SA.

Figure 2.4 Virulence Factors for ETEC.

2.6 Characteristics of AIEC

The AIEC thought to be potentially causative in CD have the general colonisation factors, type 1 pili and mucinases described in section 2.4, but may also have increased numbers of specific virulence factors which determine their tropism. One such factor has been identified - long polar fimbriae (lpf) (258), which may facilitate the translocation of AIEC across m-cells overlying Peyer's patches and lymphoid follicles, of key interest as the earliest lesions CD often occur at the ileum. The stress 'high temperature requirement' protein (HtrA) (259), and the disulphide bond forming (DsbA) protein (260), which facilitates cytoskeletal change have been demonstrated to be important for the replication of one strain of AIEC within macrophages (strain LF-82), which follows translocation. In contrast some genes may be present in much smaller numbers in CD *E.coli* in comparison to control *E. coli*, such as the iron uptake chelate (*iucD*) gene, which is involved in the synthesis of the iron siderophore aerobactin. It is also possible that the differences between the virulence genes utilised by AIEC and 'standard' *E. coli* are small, and some studies have not identified a significant difference in virulence factors between AIEC and control *E. coli*. The bacterial genes encoding virulence in AIEC bacteria warrants further evaluation as they may provide utilisable therapeutic targets (261).

2.7 *Mycobacterium avium* subspecies *paratuberculosis* (MAP)

MAP has been a putative causative organism for CD for almost a hundred years and is a continuing source of controversy (262). Johne's disease in cattle, caused by *Mycobacterium paratuberculosis* and CD have similarities. Reported detection rates for *paratuberculosis* DNA in CD tissue range between 0 and 100% (47). Meta-analysis of published studies shows a pooled risk difference ('risk' of presence of MAP in control patients subtracted from 'risk' of presence of MAP in CD patients) of 0.23 (95% CI 0.14 – 0.32) (263). Laser dissection of CD tissue has identified MAP DNA within granulomas but not within distant non-granulomatous tissue which might explain some of the discrepancies (205). MAP has been shown to release mannose-containing glycoconjugates that inhibit bacterial killing by macrophages *in vitro* (208) and an indirect role for MAP in CD pathogenesis is plausible.

The link between MAP and IBD, assessed by presence of IS 900 (a MAP DNA insertion element) in Crohn's and control tissue PCR, and enzyme-linked immunosorbent assay (ELISA) of serum immunoglobulin reaction to MAP, have also been examined in another recent meta-analysis. This produced a pooled odds ratio of 7.01 (95% CI 3.95-12.4) for PCR, and 1.72 (95% CI 1.02-2.90) for ELISA when comparing CD and controls (124), but proof of a causal role needs further work. A recent controlled trial of anti-MAP therapy in CD has been disappointingly negative (264). Moreover anti-TNF therapy can cause reactivation of *Mycobacterium tuberculosis* and its efficacy in CD is surprising if MAP is a significant pathogen.

2.8 The mechanism of interaction of AIEC with macrophages

Macrophage function and response to commensal bacteria, particularly adherent invasive *E. coli* AIEC, may be pivotal in CD. Genome wide associations with CD include macrophage defects and are discussed in section 1.5.1, and the associations of *E.coli* with macrophage tissue in CD in section 1.8.3. The internalisation of bacteria into macrophages, and the enzymatic processes and autophagy machinery required to eliminate bacteria are key patho-physiological events, which may be orchestrated by signalling molecules. These molecules may represent novel therapeutic targets (265). Cell signalling events following internalisation of *E.coli* and their sequelae (206) result in the inflammation, structuring and ulceration seen in CD tissue. Defects in the autophagy process within macrophages may be responsible for the pathology of CD, and in-vitro work has demonstrated that there is enhanced *E.coli* replication within the autophagolysosomes of cells which are deficient in the autophagy proteins IRGM and ATG16L1 (266).

One of the histological features of CD is the presence of a coalescence of macrophages or granulomata. Whilst the granuloma is not pathognomonic of CD, its presence within tissue is supportive of the diagnosis, and in one series granulomata were seen as frequently as 1/4 biopsies from CD tissue, with tight well defined appearance discriminating these from the granulomata seen in tuberculosis (267). The prevalence of these lesions microscopically is supportive evidence for their importance in CD. Macrophages utilise a number of mechanisms for killing bacteria including production of reactive oxygen species (259) and encapsulation of these bacteria within phago-lysosomal intra-cellular pockets (208;242;265) and the putative pathogenic AIEC in CD adopt a number of strategies to avoid this. The alkalinisation

of these subcellular phagolysosomal organelles is also known to enhance bacterial killing (268). The ability of bacteria, particularly *E coli*, to survive and replicate within these macrophages (206;242) may be what governs their pathogenicity.

2.9 Antibiotics in IBD

If CD results from inadequate clearance of mucosal bacteria then use of antibiotics would be predicted to have beneficial effects. The gut microbiome can be altered by antibiotics and these have been evaluated in a number of clinical trials (269).

Choice and combination of antibiotics is key and should target the suspected bacterial pathogens in CD. Many studies are not blinded or placebo controlled and can involve other treatments making comparisons difficult. Rifabutin, clarithromycin and clofazimine (targeting *M. paratuberculosis*) have shown improvement over placebo at 16 weeks, but this was not sustained (47). A meta-analysis of broad spectrum antibiotics, involving six randomised and blinded trials, in patients with active CD (270) demonstrated an advantage for antimicrobial therapy, particularly when ciprofloxacin was used, and recent meta-analysis studies looking at pooled outcomes for antibiotic therapy showed statistical significance, even if antibiotics were not advocated based on an inability to clearly specify which antibiotics were of most use (269).

The more recent meta-analysis of antibiotic trials in IBD divided the analysis into sections assessing their usefulness in CD and UC separately, as well as their use for both maintenance of remission as well as induction of remission. Analysis of 9 pooled RCT showed that the use of antibiotics had statistical significance for the induction of remission in UC, though it was not clear which antibiotics were most

important. Single antibiotics or combinations of ciprofloxacin, tobramycin, rifaximin, and vancomycin were used with pooled data showing significance (269;271-275). There is a real paucity of data evaluating antibiotic use in maintenance therapy for UC with one trial showing no effect (276).

Trials comparing the effect of antibiotics for the induction of remission in CD following relapse found a statistically significant effect for antibiotic therapy in induction of remission in CD. This was for the pooled analysis of 1160 patients, though again it was not clear which specific antibiotics were beneficial as a diverse number of antibiotics were used alone or in combination (264;269;277-282). Similarly there was some benefit in improvement of fistula drainage with use of ciprofloxacin or metronidazole (283). Similar improvements were seen in maintenance treatment of CD with antibiotics, in general targeting *Mycobacteria* (264;269;277), and it may well be that the selection of antibiotics targeting pathologically relevant micro-organisms in IBD, coupled with a favourable side effect and resistance profile for the antibiotics, may be a more useful clinical approach.

The susceptibility of CD *E. coli* within macrophages to antibiotics has been evaluated *in vitro* (242). Ciprofloxacin, rifampicin, tetracycline, sulfamethoxazole, clarithromycin, and trimethoprim were all associated with kill rates greater than 50% at C_{max} , the published peak serum concentration of antibiotic achievable in patients taking a conventional dosing regimen. A combination of ciprofloxacin, tetracycline, and trimethoprim resulted in a 97% kill rate versus 86% for ciprofloxacin alone when assessed at 10% C_{max} . Further studies are required, with appropriately targeted antibiotic therapy, particularly in combination.

2.10 The use of immunomodulators in clinical practice and their possible effects on macrophages and bacteria *in vitro*

It follows that if replication of *E. coli* within macrophages is central to disease activity then inhibition of this replication should result in clinical improvement. Drugs which are classically considered to be immunomodulators, such as azathioprine and 6-mercaptopurine (6-MP), the metabolic intermediate of azathioprine (see figure 2.5), are often used as a therapy to maintain clinical remission in CD and UC, with steroids such as hydrocortisone and prednisolone commonly used to induce remission (284).

2.10.1 Azathioprine, 6-MP and 6-thioguanine (6-TG)

The effects of azathioprine, 6-MP, and 6-TG, on replication of CD specific *E. coli* strains within macrophages are unknown. Effects of these drugs on the replication of other bacteria has been seen, with Azathioprine and 6-MP known to inhibit MAP replication, with some synergism seen with macrolide antibiotics (285;286). The different effects of azathioprine and 6-MP on the activity of the Nitric Oxide Synthetase (NOS) enzyme using *in vitro* macrophages has been previously examined (287) but effects on *E. coli* replication have remained unexplored. Similarly the effects of the main end metabolite of azathioprine, 6-TG, have not been evaluated in terms of its effects on modulation of *E.coli* replication within macrophage function, though it is known to have a direct bactericidal effect on *E. coli*. as well as other bacteria when used in nanoparticle treatments (288). It is also known that the intercalation of 6-TG into macrophage cell DNA makes the cells more sensitive to reactive oxygen species (ROS) produced endogenously or in cocultures, so this

metabolite's effects on intra-macrophage *E.coli* replication were also investigated in this work (chapter eight).

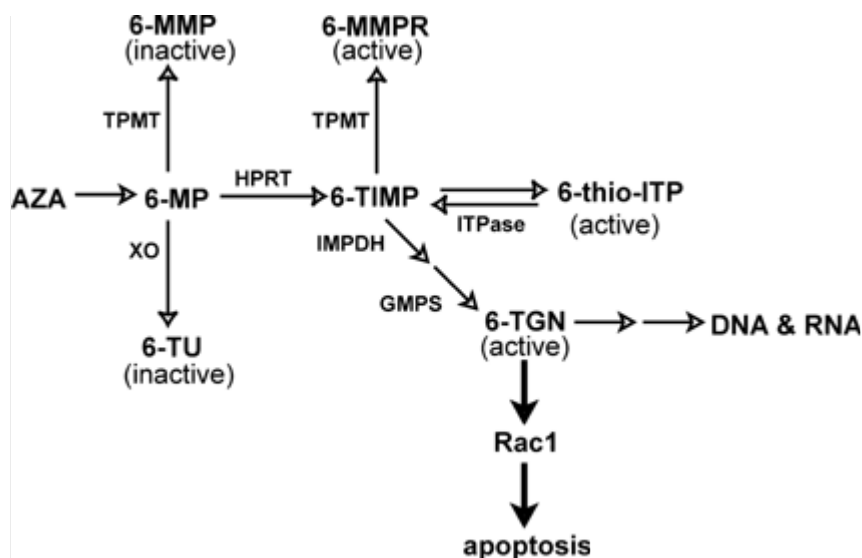


Figure 2.5 Illustration of the Metabolism of thiopurines.

Metabolism of thiopurines. The pro-drug azathioprine (AZA) is converted rapidly to 6-mercaptopurine (6-MP), which is metabolised via three competing pathways, mediated by xanthine oxidase (XO), thiopurine S-methyltransferase (TPMT), and hypoxanthine guanine phosphoribosyl transferase (HPRT). The reaction mediated by XO produces the inactive metabolite 6-thiouric acid (6-TU). TPMT catalyses the production of an inactive metabolite (6-methylmercaptopurine, 6-MMP). The HPRT reaction generates 6-thioinosine monophosphate (6-TIMP). 6-TIMP may itself be transformed into thioguanine nucleotides (6-TGN) by the rate limiting enzymes inosine-5-monophosphate dehydrogenase (IMPDH) and guanine monophosphate synthetase (GMPS), methylated into 6-methylmercaptopurine ribonucleotides (6-MMPR) by TPMT or phosphorylated to become 6-thio-inosine triphosphate (6-thio-

ITP). 6-MMPR, 6-TGNs, and 6-thio-ITP are all active metabolites. The accumulation of 6-thio-ITP to toxic levels is prevented by inosine triphosphatase (ITPase), which converts 6-thio-ITP back to 6-TIMP. AZA can act as an immunosuppressant via the random incorporation of 2'-deoxy-6-TGTP into DNA and RNA. Modified from (289).

2.10.2 The effects of steroids on macrophage function

Steroid therapy in CD and UC is frequently used in relapse to control symptoms, but does not affect the overall natural history of these diseases, and in fact their use can be associated with adverse prognosis over a patient's lifetime (14;20). The effects of dexamethasone on macrophages have been previously investigated, and it has been shown to have a suppressive effect on both phagocytosis via inhibition of the phospholipase-A2 enzyme (290;291) and the level of activity of inducible nitric oxide synthetase (iNOS) (292), both of which would seem to inhibit intra-macrophage bacterial killing. However, work with human monocyte-macrophages has demonstrated a phagocytosis stimulating effect with dexamethasone (293), which needs clarification. Hydrocortisone has previously been shown to inhibit monocyte recruitment from peripheral blood, and peritoneal macrophage numbers in mice (294), but the drug's effects on *E. coli* replication within macrophages have not been explored. The effect of this steroid at a clinically relevant concentration was also investigated in this work in chapter eight.

2.11 Prebiotics, probiotics and soluble fibre

Prebiotics are ‘food ingredients fermented by intestinal bacteria that selectively promote changes in the gut ecosystem’ (295). Inulin and oligofructose stimulate saccharolysis in the colon and enhance growth of *lactobacilli* and *bifidobacteria*. This effect has been associated with reduced mucosal inflammation in several animal models of IBD (295). Preliminary clinical trials have evaluated inulin in pouchitis (a condition in which the ileal pouch becomes inflamed after colectomy and reconstructive ileal pouch-anal anastomosis for UC) and oligofructose in UC and CD, generally involving patients with mild to moderate disease, and inulin improved endoscopic and histological appearance in pouchitis (295). The other trials suffered from low numbers or absence of a control arm (in the CD study) and were inconclusive. A recent adequately powered randomised double-blind RCT examining the effect of fructo-oligosaccharides on active CD was negative (296), but further studies examining other prebiotics, or combinations of prebiotics are required.

Probiotics have been assessed in animal models and some clinical trials (297). However, there are few large randomised double blind clinical trials. The probiotic VSL#3 reduced pouchitis relapse rate by 85% in one study and there is some suggestion that it can have an effect in maintaining UC in remission. Some strains of *E.coli* may have a beneficial effect on intestinal health, and are utilised therapeutically. *E. coli* nissle 1917 has been shown to promote synthesis of inducible human beta-defensins in cell lines (298) and has a maintenance effect in UC (299;300).

It may be that probiotics and prebiotics can be used as co-therapies (synbiotics) in genetically bacteriologically susceptible patients if further evidence accumulates.

Currently there are more studies showing beneficial effects of probiotics in prevention of inflammation rather than treatment of inflammation. Apical TLR9 stimulation of intestinal epithelial cells delivers negative signals that attenuate pro-inflammatory signalling induced by basolateral epithelial cell stimulation by TLRs (301) and 'hyper-polarise' the epithelial cell. Probiotics could work in this way. Basolateral TLR5 and TLR9 activation induce IL-8 and NF κ B release respectively, and inflammatory models demonstrate that antigens may access basolateral sites (214;302), which may occur particularly when pro-inflammatory cytokines are present in the mucosa, and junctional protein internalised (212). It may be that the contrasting effects of basolateral versus luminal stimulation of TLR9, and the potential for flagellin released from probiotics to access basolateral TLR 5 in inflamed mucosa might explain the differences seen with probiotic treatment used prophylactically versus post-inflammatory.

Our group has been investigating the hypothesis that bacteria-epithelial interactions, many of which may be lectin-carbohydrate receptor-mediated, might be inhibited by soluble plant fibres. A number of fibres, including particularly soluble plantain (banana) fibre, have been shown to inhibit adhesion of *E. coli* to the intestinal mucosa (232). This has obvious potential therapeutic benefit in arresting the events that induce mucosal inflammation in CD. In areas of the world where plantain fibre is consumed in large amounts, (Africa, India and the West Indies), the prevalence of CD is rare. It is possible that other foodstuffs or fibres could inhibit or promote (western

diet) bacterial adherence, and this merits further evaluation. The interaction of fluorescently labelled bacteria with intestinal cells has been investigated by the FACS technique (303) and affords the possibility of testing novel therapeutic foodstuff interactions with bacteria and epithelial cells with this modality *in vitro* (see chapter nine).

Figures 2.6 and 2.7 below show a current view of the possible pathogenesis of CD and UC respectively, with potential therapeutic targets identified in figure 2.6.

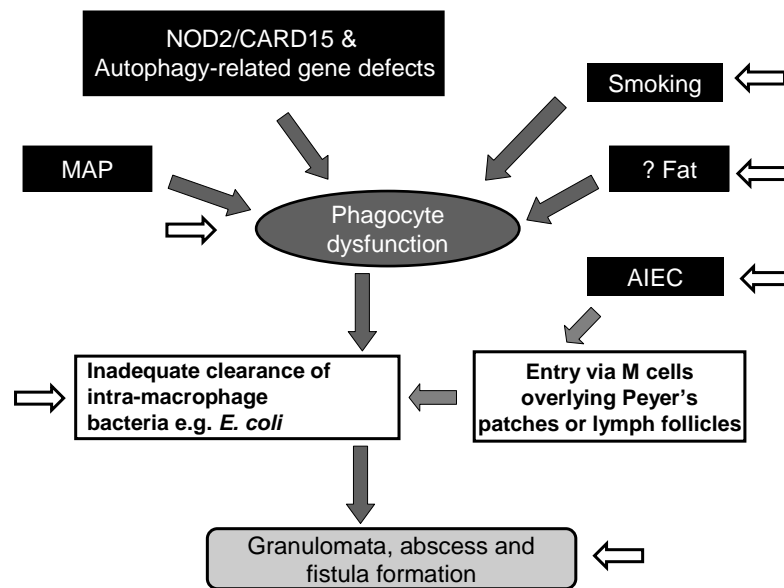


Figure 2.6 Gene–environment interactions in CD: defining new therapeutic targets. Defective phagocyte dysfunction could result from CD-associated genetic mutation, environmental factors and *Mycobacterium paratuberculosis* (MAP) wall components. Ingested microbiota, such as AIEC, may be presented to macrophages by M cells of the FAE overlying Peyer's patches of the small intestine and lymphoid follicles of the colon. The typical early lesions in CD arising at the FAE could result from inadequate clearance of ingested gut microbiota by dysfunctional intestinal macrophages. Each white arrow represents a possible target for therapy. Most current treatments target the final step in the pathway. From (215)

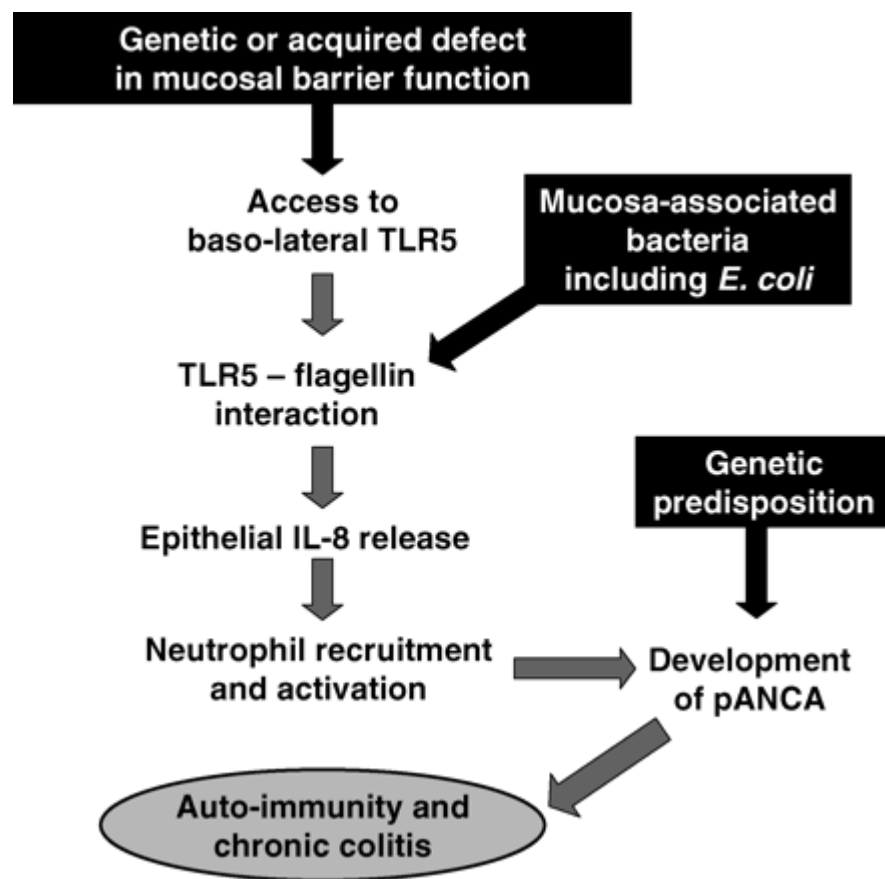


Figure 2.7 A model for bacterial involvement in UC. Current evidence is compatible with UC being an autoimmune condition, perhaps triggered by an inflammatory response to mucosa-associated gut microbiota. Shed flagellin, that elicits epithelial pro-inflammatory IL-8 release, may gain access to the basolaterally located TLR5 by a pre-existing barrier defect in patients with UC. This inflammatory response could then be perpetuated by autoimmunity. A distinct subset of pANCAs, that cross-react with bacterial antigens and are present in about two-thirds of UC cases, could have a direct role in pathogenesis (47). From (215)

CHAPTER THREE : HYPOTHESES

- 1a) Mucosa-associated *E. coli* may have a role in the pathogenesis of CD.
- 1b) Mucosa-associated *E. coli* are therefore likely to be increased in association with mucosal ulceration in CD.
- 2) The quantified levels of *E. coli* in Crohn's tissue may correlate directly with levels of mucosal inflammation seen macroscopically or microscopically, or even with clinical scores of disease activity.
- 3) The aphthous ulcers of CD patients, which are likely to represent the earliest lesions, may be populated by bacteria (possibly *E. coli*) that have an important role in pathogenesis.
- 4) Drugs may have efficacy in CD if they can kill *E. coli* within macrophages. These may include antibiotic combinations but also may include some of the drugs that are commonly used in CD maintenance therapy including immunosuppressives.
- 5) The adherence of *E. coli* and other diarrhoea-causing bacteria to intestinal cells may be modified by dietary components, including soluble non starch polysaccharides (NSP) such as those present in plantains (*Musa* [banana] *spp.*) as well as oat NSP and apple pectin.

CHAPTER FOUR : AIMS

- 1) To develop culture-independent molecular methods of quantifying bacteria from biopsy tissue so that these can be (a) correlated with the clinical and histological status of a patient with CD (b) used to assess response to therapy with soluble fibre supplements.
- 2) To assess the quantity of *E. coli* and other bacteria within aphthous ulcers of CD patients.
- 3) To evaluate the effect of antibiotics and immunomodulators used at clinically relevant concentrations on *in vitro* replication of CD *E. coli* within macrophages.
- 4) To assess, using several *in vitro* modalities, whether dietary soluble fibre (Non-starch polysaccharide, NSP) and other dietary components affect the adherence of diarrhoea-causing bacteria and *E. coli* to intestinal cell lines.

CHAPTER FIVE : GENERAL MATERIALS AND METHODS

5.1 Mammalian Cell culture

5.1.1 Caco2 cells

To study bacteria-host intestinal epithelium adherence and invasion, the human colorectal adenocarcinoma cell-line Caco2 (#86010202) was obtained from the European Collection of Animal Cell Culture (Public Health Laboratory Service, Wiltshire, UK). Caco2 cells were grown and maintained in supplemented Dulbecco's modified Eagle's medium (DMEM), see appendix six

Caco2 cells were used when they were at a maximum of 80% confluent, and were passaged at a ratio of one in four twice weekly, using either a 10% (v/v) solution of (0.5 mg/ml) trypsin in sterile phosphate-buffered saline (PBS). For experiments (see chapter nine), Caco2 cells were initially seeded into 24-well tissue culture plates (Corning/Costar, High Wycombe, UK) at 1×10^5 cells per well and grown overnight in Dulbecco's modified Eagle medium (without antibiotics). The monolayers were then washed twice with sterile PBS before the addition of bacteria (see chapter nine).

For FACS experiments Caco2 cells were maintained until 80% confluent in flasks. 24 h before adherence assays, DMEM medium was removed, cells were washed three times in cold PBS, and cells were incubated with antibiotic-free medium. Cells were then washed three times again with PBS and removed from

tissue-culture flasks with a trypsin-PBS dilution (10% v/v), counted using a haemocytometer and adjusted to 1×10^5 cells/ml using antibiotic free DMEM (see chapter nine). The non-starch polysaccharides (NSP) used in adherence and FACS experiments in chapter nine with Caco2 cells, were extracted from plantain, the banana family (*Musa* spp.) and oats. These were provided as water soluble freeze dried powders by Provexis Plc (Windsor). Apple pectin used in the adherence experiments was supplied as a viscous solution by Sigma-Aldrich (Dorset, UK). FACS measurements were performed using a FACScan flow cytometer (Becton Dickinson, Oxford), and the data were analysed with the Cell Quest software programme (Becton Dickinson).

5.1.2 J774-A1 macrophages

To study the effect of antibiotics and immunomodulators on intra-macrophage replication, the murine macrophage-like cell-line J774-A1 (#85011428) was also purchased from the European Collection of Animal Cell Culture (Public Health Laboratory Service). This is a BALB/c monocyte macrophage cell line from the ascites of a female mouse. Human cells were not used due to previous difficulties in their extraction and maintenance at the laboratory. Cells were maintained in supplemented Roswell Park Memorial Institute RPMI-1640 medium, see appendix six. All mammalian cells were maintained at 37° C in a humidified atmosphere of 95% air, 5% (v/v) CO₂. Culture medium and supplements were supplied by Sigma-Aldrich excepting FBS (Invitrogen; Paisley, Scotland).

J774-A1 cells were grown until 80% confluent and passaged following gentle physical removal using cell scrapers (Corning® Chorges, France. Product number

#3011). Both Caco2 and J774-A1 cells were used at passage numbers less than 10 in experiments. J774-A1 macrophages were seeded into 24 well plates at the same concentration 1×10^5 /ml with 1 ml added, and used for experiments after 24 or 48 h (see chapter eight). When macrophages were treated with drugs, antibiotics were solubilised in dimethyl sulfoxide (DMSO) followed by dilution in RPMI and then sterile filtration. Immunomodulators were also solubilised in RPMI media before sterile filtration and all drugs were then prepared at the experimental concentrations specified in chapter eight using RPMI as diluent. Gentamicin was an exception as it was supplied as a liquid. All drugs were supplied by Sigma-Aldrich.

5.1.2.1 Assessment of possible cytotoxic effects of pharmacological agents on macrophage cells

Potential cytotoxic effects of pharmacological agents used in this thesis on macrophage cells were assessed. This involved both cell counting using a haemocytometer and the use of the Toxilight™ adenylate kinase assay kit (Cambrex, Lockland USA). Cell supernatants were evaluated as per manufacturer's instructions (protocol 2, of kit LT07 – 217). Techniques used are described in chapter eight.

5.2 Bacterial strains

5.2.1 Adherent-invasive *E.coli*

Bacterial strains examined for the presence of the *uidA* gene included mucosally-associated *Escherichia coli* (*E. coli*) isolated from colonoscopic biopsies of

patients with Crohn's disease (CD) attending the Royal Liverpool University Hospital by Helen Martin (232). These were designated 'HM' and specifically were HM95, HM154 (non adherent, nonhaemagglutinating strains), HM580, HM605, HM615, HM670 (adherent haemagglutinating strains). Two further *E. coli* isolates were kindly donated by the research group of Professor Arlette Darfeuille-Michaud, (Clermont Ferrand): LF10, an adherent, invasive haemagglutinating strain, and LF82 an adherent, invasive non-haemagglutinating strain, both isolated from patients with ileal CD (200;206). The majority of these *E.coli* studied possess ability to adhere to, and invade Caco2 cells (200;232) and to survive and replicate within phagolysosomes of macrophages (206;242) and meet the phenotypic criteria of the pathotype designated as Adherent-Invasive *E.coli* (AIEC) (207).

Further processing of the HM605 strain for quantification standards is discussed in section 5.2.4

5.2.2 Control bacterial strains

Given that *uidA* is a gene unique to *E. coli* or *Shigella* species and not found in other bacteria a negative control for *uidA* was included: *Klebsiella pneumonia* (304). (HM534) was obtained and characterised from a patient in (232) and PCR negativity for the bacterium was also confirmed using the NCBI database.

Two further bacteria *Enterococcus faecalis* (HM65) and *Citrobacter rodentium* (HM481) also isolated in (232) from colonic samples were also used to provide

negative genomic DNA controls for *uidA* qPCR in (Standardisation method 3) with DNA extracted as per section 5.3.1.

Two probiotic bacteria *Lactobacillus paracasei* and *Bifidobacterium longus* were used as negative control template in standardisation method four. Each was cultured as per *E. coli* strains on Luria Bertani (LB), agar (see appendix six) overnight to stationary phase and genomic DNA extracted as per section 5.3.1.

K12, an indolent laboratory strain of *E. coli* with limited *in vitro* adherence was used as a reference negative control as per (242) in the experiments evaluating diarrhoeal pathogens in chapter nine.

All bacterial isolates were stored on Protect beads (Scientific Laboratory Supplies, Yorkshire, England) in glycerol broth at -80°C. Each was grown overnight on LB agar at 37°C.

5.2.3 Diarrhoeagenic bacterial strains

Diarrhoeagenic *E. coli* used for studies to examine the inhibitory effects of foodstuffs on bacterial adhesion included enteropathogenic (EPEC) strains D55 and E2348/69 which were obtained from stocks held within the Department of Clinical Infection, Microbiology and Immunology, University of Liverpool. Enterotoxigenic *E. coli* ETEC C410 (serotype O160, ST+ and LT+) was kindly supplied by Dr Godfrey Smith (Medical Microbiology, Royal Liverpool & Broadgreen University Hospitals NHS Trust, UK). All enteropathogenic *E. coli* were cultured on LB agar plates with overnight incubation in air, at 37°C. *C. difficile* Type 027 (strain 080042), also

supplied by Dr. Godfrey Smith, was grown on Fastidious Anaerobe Agar (Lab M Ltd, Bury, UK) under anaerobic conditions. *Campylobacter jejuni* (strain 11168H (305)) had also been prepared within the Department of Clinical Infection, Microbiology and Immunology, University of Liverpool. They were cultured in a micro-aerophilic environment for 48 hours before use on the day of experimentation.

5.2.4 Use of the *E. coli* strain HM605 in standardisation methods 1-3.

After overnight growth on LB agar, *E. coli* HM605 cells were diluted in PBS to an absorbance (550 nm) of 0.825 (1×10^9 *E. coli*/ml).

When standardisation required quantification of colony forming units (CFU), after each dilution of the initial bacterial suspensions, seven further serial 1:10 dilutions were performed in a 96 well plate.

Based on standard curves for *E. coli* HM605 established within our laboratory, up to four dilutions were selected to provide countable colonies after plating. 50µl of bacterial suspension were plated out on LB agar in triplicate and incubated at 37°C for 24 h before colonies were counted and standardised per ml. CFU numbers were calculated using the mean of triplicate measures at two dilutions (except for the most dilute initial suspension, where only one dilution was useful). As a control, sterile PBS alone was plated out neat in triplicate.

5.3 Human and Bacterial DNA Extraction

Bacterial and human cell DNA was extracted in a number of ways, which included both a crude technique involving boiling, vortexing and centrifugation, as well as commercial DNA extraction kits:

5.3.1 Crude DNA extraction

Crude genomic DNA template extracted from bacterial suspensions was used for endpoint PCR, primer optimisation experiments in the development of a qPCR bacterial quantification method and in the preparation of control bacterial DNA for qPCR quantification methods three and four (all chapter six). Briefly, following overnight growth on LB agar (see appendix six) in the 37°C incubator, two colonies of each bacterial strain were selected and removed with an inoculating loop to 100µl sterile-filtered PCR grade water in nuclease-free tubes. DNA template was prepared by boiling (100°C for 10 min), vortexing for 1 min and centrifugation (13,000 rpm, 13,200 g for 10 min) of these suspensions. DNA template was diluted as required for each standardisation assay.

5.3.2 Qiagen mini-prep purification of bacterial and human DNA

This kit was used for extraction of bacterial DNA, human DNA, and the combination of both in human biopsies.

One ml of a suspension of *E. coli* was prepared to an absorbance (550 nm) of 0.825, equivalent to 1×10^9 organisms/ml, and used for Qiagen DNA mini-kit (51304)

extraction (306). The extraction was performed using the Qiagen tissue kit protocol for bacterial DNA extraction (appendix D of manufacturers protocol). After overnight growth bacterial pellets were resuspended, homogenised with buffer ATL, treated with proteinase K, 4 µl of 100 mg/ml RNase A (Sigma-Aldrich) buffer AL, 100 % ethanol, AW1 and AW2 (see appendix six for buffer composition), before elution of DNA from QIAamp mini-spin columns with PCR grade water (Roche). Recovery was with only one elution volume (50 µl) for all extractions.

When human biopsies were processed from the clinical studies the Qiagen DNA kit was also used. The human samples (see section 5.11 and chapter six) were thawed. Initially the biopsies and the bacterial pellets were treated differently. Whilst bacterial pellets were resuspended in 180 µl buffer ATL as before in a 1.5 ml microcentrifuge tube, the biopsies were also mechanically homogenised. Each biopsy was placed in 80 µl PBS in a 1.5 ml tube and then homogenised, using the laboratory nucleic acid homogeniser probe (Fisher, Loughborough, UK) until complete homogenisation. In between samples the homogeniser probe was washed with 70% (v/v) ethanol and PBS. 100 µl of buffer ATL was then added to the sample. Both the bacterial pellet and biopsy were processed identically from this point.

The DNA extraction was completed as above from the proteinase K step, again with recovery by a single elution. These DNA elution samples were stored at -80°C.

5.3.3 Use of the Promega kit to extract and optimise bacterial DNA

The Promega kit gives a high yield of bacterial DNA from usual starting materials, 100 µg from 5 ml of bacterial suspension (page 4 of the Promega Wizard® Genomic DNA Purification Kit (A1125) protocol). This was therefore used to enhance *E.coli* bacterial DNA template prior to qPCR quantification methods three and four in chapter six.

The *E. coli* strain HM605, a typical AIEC with *uidA* PCR positivity was used to generate *E.coli* DNA, and was grown overnight in standard LB broth at 37°C before use. Six ml cultures were harvested by centrifugation (13,000 g for 2 min), then processed according to the Promega Kit instructions (section G - isolating genomic DNA from Gram negative bacteria). Briefly, bacteria were lysed in nuclei lysis solution at 80°C then RNase I-treated. Protein was removed with protein precipitation solution and finally DNA was precipitated using 70% ethanol, and rehydrated with DNA rehydration solution respectively. DNA was stored at -80°C.

5.4 Endpoint PCR amplification reactions

Two µl aliquots of supernatant from the centrifugation of crude genomic DNA preparations of AIEC bacteria from CD patients were used in 50 µl PCR reactions which contained 45 µl of 1.1X ABgene PCR mastermix (Advanced Biotechnologies Limited; Surrey, UK) and 1.5 µl each of forward and reverse *uidA* gene primers (1.0 µM final concentration, *uidA F1* and *uid R1* (pair one)) – see table 5.1). Reactions were carried out on the Techne Genius Thermal Cycler (Rhys Scientific; Chorley, UK). An initial denaturing step at 95°C for 15 min was performed, then 35 cycles of :

94°C for 15 s, annealing temperature of 55°C for 1 min, 72°C for 1 min, with an additional extension time of 72°C for 10 min.

5.4.1 Endpoint PCR DNA electrophoresis

The products of PCR reactions were electrophoresed on 2% (w/v) agarose gels (Sigma), containing 10 µl per 500 ml agarose of 10 mg/ml ethidium bromide, for 50 min at 100 V. Gel images were acquired using the 'Quantity one' software package (BioRad, Hemel Hempstead, UK), on the Flu- imager (BioRad). Hyperladder IV (100-1000 bp) (Bioline, London, UK) was used to define product size.

5.5 The use of spectrophotometry to quantify DNA

DNA was quantified by standard spectrophotometry. Absorbance at wavelength 260 nm was multiplied by 50 to quantify DNA in µg/ml. The ratio of absorbances at wavelength 260 nm/280 nm was used to assess DNA purity (with a target range for this ratio between 1.7-1.9) using samples in standard semi-micro UV cuvettes (10 mm path length); (CM scientific, Silsden UK).

Samples were also quantified using the Nanodrop™ spectrophotometer (Thermoscientific, Loughborough, UK) for comparison. The Nanodrop™ has advantage that only 1-1.5 µl sample is required for quantification (as opposed to 50 µl in standard UV cuvettes), and has increased sensitivity for measuring DNA concentration with stated precision of 1.5 ng/µl.

5.6 Oligonucleotide primers used in the PCR reactions performed in this study

Primers and probes used are listed in Table 5.1. Supplied by Eurogentec (Belgium).

Table 5.1 Primers used in PCR assays, all sequences specified in 5'-3' direction

Primer Name	Sequence (5' to 3')	Amplicon size/bp	Annealing temp/°C	Reference	Target
<i>uidA</i> F1	Forward: TATGAACTGTGCGTCACAGCC	186	55	(205)	<i>uidA</i> gene
<i>uidA</i> R1	Reverse: CATCAGCACGTTATCGAATCC				
<i>uidA</i> F2	TGTGATATCTACCCGCTTCGC	61	60	(218)	<i>uidA</i> gene
<i>uidA</i> R2	CAGGAACTGTTCCGCCCTTCA				
<i>uidA</i> TaqMan® probe 2	FAM/TCGGCATCCGGTCAGTGGCA-BHQ-1				
<i>uidA</i> F3	GCCAGACAGAGTGTGATATCTACCCGT	135	55	(205)	<i>uidA</i> gene
<i>uidA</i> R3	CCACGCAAGTCCGCATCTTC				
16S F	ACTCCTACGGGAGGCAGCAGT	200	49	(307)	16S rRNA gene common sequence
16S R	GTATTACCGCGGCTGCTGGCAC				
18S F	Eurogentec (RT-CKFT-18S control kit)	121	60	(218)	18S rRNA gene
18S R					

5.6.1 Optimisation of *uidA* qPCR

The primer sequences used for *uidA* qPCR were different to those used in endpoint PCR as a shorter amplicon size improves efficiency of qPCR. Primer pairs *uidA* F2 and *uidA* R2 together with *uidA* TaqMan® probe 2 (see Table 5.1) were used to quantify *E. coli uidA* genes as previously described (218). For optimisation assays of primers, aliquots of a genomic DNA sample extracted from a bacterial suspension (the equivalent of DNA crudely extracted from 1×10^7 *E. coli*/ml) were used as a ‘constant’ template, with TaqMan® probe final concentration constant at 200 nM, while primer final concentrations were varied in combinations of 50 nM, 300 nM and 900 nM to see which produced the lowest threshold cycle number ‘Cp’ and highest fluorescence in unit time. qPCR composition and reaction conditions are described in 5.7.1-5.7.2

5.7 qPCR Reaction Conditions and a summary of primers used in quantification processes

Throughout qPCR assays, pipettes were treated for two hours pre- and post assay with UV light. This was to minimise contamination and qPCR amplification crossover. Table 5.2 summarises which of the primer pairs from table 5.1 were used in endpoint PCR, primer optimisation and which specific quantification assays, which are discussed in chapter six.

Table 5.2 Summary of primers (from table 5.1) used in specific assays and quantification methods in this thesis

	PRIMER PAIR USED			
ASSAY	<i>uidA</i> F1 R1	<i>uidA</i> F2 R2	<i>uidA</i> F3 R3	16S
Endpoint PCR	YES	NO	NO	NO
Optimization of qPCR primer concentrations	NO	YES	NO	NO
qPCR quantification method 1	NO	YES	NO	NO
qPCR quantification method 2	NO	YES	NO	NO
qPCR quantification method 3	YES	NO	NO	NO
qPCR quantification method 4 (non-linear plasmid DNA)	YES	YES	NO	YES
qPCR quantification method 4 (linear plasmid DNA)	YES	YES	YES	YES

Sections 5.7.1 onwards detail the reaction composition and conditions used for each primer pair in Table 5.1. *uidA* primer pairs F2 and R2 were used initially in optimisation assays with crudely extracted genomic DNA as described in the section above to ascertain their optimal concentrations.

5.7.1 qPCR reaction composition for *uidA* F2 and R2 primers and probe

PCR reactions, typically in 20 µl volumes, were performed in triplicate for each crude DNA template in a LightCycler 480® (Roche, West Sussex, UK), contained 5 µl template, 10 µl LightCycler 480® probes mastermix (Roche), 1 µl of probe (200 nM final concentration) and each primer (50-900 nM final concentration), and 2 µl PCR grade water (Roche). The non-template control was 5 µl of PCR grade water (Roche). Where primers or probe were not added (as controls), equivalent volumes of PCR water were added instead.

Following optimisation *uidA* F2 and R2 primers were used at 900 nM concentration, as were the other primers used and the *uidA* probe was used at a 200 nM concentration.

5.7.2 Thermocycler conditions for *uidA* gene amplification with primers F2 and R2

Reactions were carried out in the LightCycler 480®, on the first occasion with pre-incubation temperatures of 50°C for 2 min followed by 94°C for 10 min, then 40 cycles of 94°C for 20 s, 55°C for 20 s, then 72°C for 30 s, as described previously (218) but with a short cooling step added (40°C for 30 s). Fluorescence was measured at each 72°C step.

This was modified on advice from the manufacturer of the LightCycler 480® machine, with fluorescence acquisition at 60°C (probe annealing step), a single pre-incubation step (95°C for 10 min) and 40 amplification cycles- 95°C for 10 s, 60°C for 30 s and 72°C for 1 s, with a final cooling step 40°C for 30 s. These changes did

not affect fluorescence signal adversely, and were used as standard conditions for primers *uidA* F2 and R2 subsequently.

5.7.3 qPCR reaction composition and reaction conditions for *uidA* primers F1 R1 and F3 R3 (incorporating SYBR green technology)

qPCR reaction compositions for these primer pairs were as the standard mix (5.7.1), but instead contained 10 µl LightCycler 480® SYBR green probes mastermix (Roche) and 3 µl PCR grade water (Roche) as the 1 µl *uidA* probe was not used.

The reaction conditions (modified from (205)) were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 55°C for 20 s, 72°C for 30 s (with fluorescence acquisition), followed by a melt curve analysis (95°C for 5s, 65°C for 1 min and then continuous acquisition from 97°C).

5.7.4 qPCR of non-linearised *uidA* plasmid DNA for standard curves.

After initial experiments using crudely extracted, and commercial kit extracted genomic DNA (quantification method 1) as template, development of the quantification assay meant that modified plasmid DNA, generated using amplicons produced by the primers *uidA* F1 R1 and F2 R2 (see chapter six) were evaluated with the *uidA* primers.

Non-linearised *uidA* amplicon 1 and 2 plasmid DNA was serially diluted ten fold and used to produce standard curves using the standard conditions for *uidA* primers F1 R1 2 (5.7.3), and *uidA* primers F2 R2 (5.7.2). The non-linearised plasmid-*uidA* amplicon

1 DNA samples were assessed alongside negative control probiotic bacteria (*Bifidobacterium* and *Lactobacillus*) to confirm specificity.

5.7.5 16S rRNA qPCR assay

The reaction composition was as 5.7.3 but used 16S primers, and an additional 2 µl PCR grade water when only 3 µl of DNA template was used in the assay. The temperature conditions and timings were as 5.7.3, but with a primer specific annealing temperature of 49°C used.

5.7.6 qPCR of human 18S rRNA genomes, the denominator assay

Serial ten-fold dilutions of the human chromosomal DNA provided at 30 ng/µl (which corresponds to 10,000 genome copies of 18S), with the Eurogentec control kit (RT-CKFT-18S) were prepared. There are two hundred 18S rRNA genes per human genome (see Eurogentec technical data sheet – RT-CKFT-18S). The control used was PCR grade water (Roche). The composition of the 18S rRNA qPCR reactions was entirely different to previous qPCR apart from the template volume used, as were reaction conditions. Each 20 µl qPCR reaction (in triplicate) contained 5 µl of chromosomal DNA template (5 - 50,000 genome range, or 15 pg – 150 ng DNA), 3.4 µl PCR grade water, 0.4 µl of 18S rRNA TaqMan® probe (125 nM final concentration), 1.2 µl 18S rRNA primer mix (600 nM final concentration), and 10 µl of the LightCycler480® probes mastermix (Roche). This control kit has been used previously as a denominator (218). Amplifications were carried out in the LightCycler480® with a pre-incubation temp of 95°C for 10 min, followed by 40

cycles at 95°C for 15 s, then 60°C for 1 min, and 72°C for 1 s, with fluorescence measured at each 60°C step, with a final cooling step 40°C for 30 s. These conditions are as per 18S rRNA kit instructions, with slight modification to suit the LC480® machine and the amplicon size generated using these primers is 121bp.

5.7.7 qPCR of plasmid DNA (all forms)

5µl of plasmid DNA were used as template in qPCR amplification reactions to confirm successful insertion of the *uidA*, and 16S rRNA amplicons. Primers and reaction conditions are detailed in table 5.1 and sections 5.7.1 – 5.7.5 with a primer pair used for its corresponding amplicon (or nested amplicon)

5.8 Generation of non-linearised *uidA* and 16S plasmid DNA for quantification : Plasmid and amplicon preparation including confirmation of suitable product, ligation reactions, and transformation of cells to accept cloned plasmid-gene amplicon DNA

The generation of non-linearised plasmid DNA involved four main steps; generation of an appropriate amplicon, ligation of this amplicon into plasmid DNA, transformation of cell lines to accept these cloned plasmids, and extraction of plasmid DNA from these cells.

5.8.1 Plasmid and amplicon preparation, ligation reactions and confirmation of amplicon insertion into plasmids

pGEM® -T vector was supplied as a 3000 bp linearised plasmid (Cat.# A3600, Promega, Southampton, UK), convenient for the cloning of PCR products. The vector was prepared by cutting with a blunt-ended restriction endonuclease and adding a 3' terminal thymidine to both ends. These 3' T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products with 5' A overhangs. This was ready to accept PCR products produced by thermostable polymerases in the presence of the ligase enzyme. *uidA* or 16S amplicons were generated from genomic *E.coli* DNA using primers F1 R1 and F2 R2 or 16S primers and reaction conditions described in sections 5.7.1, 5.7.2 and 5.7.5. The amplicons were 186, 61, and 200 bp in size respectively, and were used directly from the reaction mix in ligation reactions, forming a recircularised plasmid for the non-linearised *uidA* plasmid assays.

Using the Promega ligation kit (A3600) a PCR product-plasmid ligation reaction was set up, pipette mixed and incubated overnight at 4°C comprising: 10 µl 2x ligation buffer, 1 µl pGEM®-T (50 ng), 7 µl amplified PCR product, and 2 µl of T4 DNA ligase (3 Weiss units/µl activity). As a control, 1 µl of control DNA supplied with the kit combined with 6 µl PCR water were used in place of amplified PCR product to confirm that the ligation process was successful.

Confirmation that the correct amplicon had been inserted into the plasmid vector was achieved primarily by amplifying the ligated plasmid DNA using qPCR and, in one case (the *uidA* amplicon), performing a restriction enzyme digest of the

extracted plasmid-amplicon DNA products. This was followed by DNA gel electrophoresis to confirm size.

5.8.1.1 Restriction enzyme digest of the *uidA* amplicon-plasmid DNA

The sequence of the pGEM®-T (A3600) plasmid, along with a restriction enzyme map was published in the Promega product guidance, and the sequence of the *uidA* gene was acquired from Genbank (NCBI Reference Sequence: NC_010473.1). Webcutter 2 internet software was then used to check for restriction enzymes that did not cut within the *uidA* gene sequence but did cut either side of the amplicon insert on the pGEM-T plasmid. Subsequently a restriction enzyme digest was performed with enzyme, temperature, and incubation time selected as guided by the New England Biolab catalogue. The enzymes used were *Sac*II and *Spe*I (Roche), requiring a double digest and using buffer no. 4 (Roche). These enzymes were incubated together at 37°C for 1 h with the plasmid DNA:*uidA* amplicon product, followed by heat inactivation at 80°C for 20 min (*Spe*I requires 80°C to be inactivated even if *Sac*II is inactivated at 65°C). Bovine serum albumin (BSA) was needed for the action of the *Spe*I restriction enzyme and was ten fold diluted from the original concentration of 10 mg/ml, (with buffer 4 which was itself ten fold diluted in sterile distilled water to produce 1x buffer 4), to prevent any inhibitory effect on the digest reaction. The reaction components were : 2 µl reaction buffer 4, 10 µl plasmid DNA, 1 µl of each restriction enzyme 2 µl of the diluted BSA and 4 µl of sterile distilled water. Control restriction enzyme digests were set up which were either lacking the *Spe*I enzyme, the *Sac*II enzyme, or both and these were run on the same DNA electrophoresis gel.

When a restriction enzyme was omitted, an equivalent volume of sterile distilled water was used instead.

When SYBR green mastermix was used in qPCR assays, a melt curve analysis was performed as an additional means of confirming specificity of product.

5.8.2 Transformation of cell lines

The *uidA* gene amplicons, once ligated into plasmid DNA, were used to transform XL10 Gold Ultracompetent cells (Stratagene), C4040-03 (Invitrogen TM) cells, and JM107 electrocompetent cells (supplied by Dr Joanne Fothergill and Dr Chloe James (Department of Clinical Infection, Microbiology and Immunology, University of Liverpool)).

The plasmid containing the 16S rRNA amplicon was used to transform XL10 Gold Ultracompetent cells, for use as a denominator for total bacteria. The specific transformation of each cell line is described in sections 5.8.2.1 – 5.8.2.3.

5.8.2.1 Transformation of XL10 Gold® Kan^r Ultracompetent Cells using the modified plasmids, and overnight incubation of these cells

The experimental plasmids with amplicons ligated, and control DNA, was incorporated into XL10 Gold® Kan^r Ultracompetent Cells (Stratagene 200317) using the XL10 Gold® Kan^r Ultracompetent Cells transformation protocol (Revision #074003, Stratagene). Briefly, ultracompetent cells were thawed from -80°C on ice,

and 100 µl of cells were mixed with 4 µl B-mercaptoethanol on ice for 10 min. 5 µl of ligation mix (PCR amplicon product or control DNA) were added to this, mixed, and then further incubated on ice for 30 min. These tubes were then heat pulsed in a 42°C water bath for precisely 30 s before being re-incubated on ice for 2 min. 0.9 ml of pre-heated (42°C for 2 h) Nyzatidium Bromide (NZY⁺) broth was then added to each tube which were then incubated at 37°C for 1 h with shaking. Finally, 200 µl from each tube were added to pre-prepared Amp X-Gal agar plates (see appendix six) and incubated at 37°C overnight to screen for transformants.

5.8.2.2 Transformation of One Shot® TOP 10 Competent cells, C4040-03 (Invitrogen™) with modified plasmid DNA, and overnight screening incubation

Briefly, Amp X-Gal agar plates were prepared, and pre-warmed in a 37°C incubator for 30 min. The ligation reactions were centrifuged briefly and placed on ice while vials of chemocompetent cells were thawed out on ice. 5 µl of each ligation reaction was added to each 50 µl vial of cells and mixed by gentle tapping. These were incubated on ice for 30 min followed by a precise 30 s incubation in a 42°C water bath followed by placing immediately on ice. Then 250 µl of pre-warmed S.O.C. (super optimal broth with catabolite repression or enriched bacterial growth medium) was added to each vial before vials were shaken at 220 rpm or 8.5 g for 1 h at 37°C. 200 µl from each vial were then spread on prepared Amp X-gal agar plates and stored at 37°C overnight with colonies examined for blue/white screening the following day.

5.8.2.3 Transformation of JM107 electrocompetent cells with modified plasmid DNA by electoporation of cells.

Briefly a fresh overnight culture of *E. coli* JM107 cells was grown in 5 ml of LB and then inoculated into 500 ml (or 1% dilution) of LB, and these cultures were grown with shaking at 37°C to an OD₆₀₀ of 0.35-0.5. The cells were centrifuged at 10,000 rpm or 17,000 g for 10 min in a pre-cooled centrifuge, resuspended in 500 ml of ice-cold sterile distilled water, followed by three further centrifugations, and resuspension in decreasing amounts of ice-cold sterile 10% (v/v) glycerol (500 ml, 250 ml, 2.5 ml). The volumes were then transferred to cold 1.5 ml tubes and recentrifuged at 13,000 rpm (17,000 g) before resuspension and aliquoting (150 µl volumes) prior to flash freezing in liquid nitrogen. These aliquots were stored at -80°C when not used immediately.

On the day of electroporation, 1 mm electroporation cuvettes were cooled on ice. 10 µl of ligation reaction were added to 40 µl of electrocompetent cells in the cuvette. The cells were electroporated at 25 µF, 200 Ohm, 2.5 kV (Gene-Pulser II Electroporater, Bio-Rad), followed by immediate suspension in 1 ml S.O.C and shaking incubation at 37°C for 1 h. They were then plated on amp X-gal agar to screen for successful transformants.

5.8.2.4 Blue-white colour screening of plasmid-transformed bacterial cells

The Amp X-gal agar plates with the transformed cells added were checked after overnight culture and white colonies (which had successfully incorporated plasmid

DNA) were used for further subculture, and stored as useful bacterial clones for extracting plasmid DNA. This plasmid DNA was used to produce standard curves, for bacterial gene quantification as required. Control DNA was used alongside the ligation reactions with cells to confirm that they could be transformed effectively.

5.8.3 Plasmid DNA extraction

The successfully transformed ultracompetent bacteria were used to harvest plasmid DNA. This plasmid DNA extraction was performed using the QIAprep Spin miniprep kit (Qiagen, 27104) and the associated protocol from the QIAprep Miniprep handbook. In brief, one colony of transformed cells was placed in LB broth (see appendix six) with ampicillin added at 100 mg/ml, (2 x 1.5 ml) and incubated overnight at 37°C. This 3 ml of transformed bacterial cells was centrifuged at 13,000 rpm (13,225 g) for 4 min to pellet the bacteria the next day. These were resuspended in 250 µl of buffer P1 (which had had RNase A pre-added), followed by 250 µl buffer P2, and finally 350 µl of buffer N3 (see appendix six). This was centrifuged for 10 min and the supernatant from this was added to a QIAprep column and centrifuged for 60 s. The spin column was washed with 0.75 ml of buffer PE (see appendix six) and centrifuged for 60 s x2. Finally plasmid DNA was eluted by addition of 50 µl of PCR grade water to the QIAprep column in a clean 1.5 ml microfuge tube, which was allowed to stand for 1 min before centrifugation for 1 min.

5.9 Linearisation of plasmids containing the *uidA*/16S rRNA amplicons

During method development for quantification of *uidA* and 16S rRNA genes (*E.coli*), re-linearization of the modified circular plasmids containing the gene amplicons was performed as an additional step, in order to see if assay sensitivity could be improved (see chapter six).

To linearise the pGEM-T plasmids, the restriction enzyme *RsaI* was used. The sequence of the *uidA* and *16S rRNA* gene were acquired from Genbank and Webcutter 2 internet software used to check that the restriction enzyme did not cut within the gene sequences but did cut once at the side of the amplicon insert within the plasmid.

The reaction mix comprised 2 µl *RsaI* and 2 µl buffer A (Roche) with 5 µl of the plasmid DNA and 11 µl of PCR grade water. The reaction mix was incubated at 37°C for 2 h followed by 65°C for 30 min to inactivate the restriction enzyme. The gene copy number from previous calculations was divided by 4 to reflect the 1:4 dilution of the plasmid DNA in the reaction mix, successful linearisation was confirmed by 1% (w/v) agarose DNA gel electrophoresis and the linearised template was subsequently used in qPCR.

5.10 DNA gel electrophoresis to confirm the size of amplicons, restriction enzyme products, or to demonstrate plasmid linearisation

Amplicons, restriction enzyme digests of plasmid, non-linear plasmid or ‘single-cut’ linearised plasmid DNA were electrophoresed on 1-2, and also 3.5% (w/v) agarose gels (Sigma), as appropriate for product size, intercalated with ethidium bromide or

‘gel-red’, for 50 min at 100 V. The gel images were acquired using the ‘Quantity one’ (BioRad) or ‘Genesnap’(Syngene) software packages. Hyperladders I (200 – 10,000 bp), IV (100 – 1000 bp), and V (25 – 500 bp), were used to define product size (Bioline).

5.11 Small and large intestinal biopsy handling procedure used in the plantain clinical trial and aphthous ulcer study

Intestinal samples were taken in both the aphthous ulcer study and plantain clinical trial in this work, and local ethics approval was granted for both. For the aphthous ulcer study local ethics approval was from Northwest 2- Liverpool Central, reference 10/H1005/25, with the same committee separately approving the plantain clinical trial EudraCT number: 2006-005526-23, Ethics committee reference : 07/H1002/105, ISRCTN number 62819212.

Five patients were recruited to the aphthous ulcer study, in which twelve small and large intestinal biopsies in total were collected in addition to routine samples. In the plantain trial twelve patients were recruited to the biopsy arm of the RCT in which four sigmoid colon biopsies were taken in addition to routine samples.

5.11.1 Processing of samples within the endoscopy unit

An individual sample identity number was used for each intestinal sample taken from a patient, and was the only identifying number stored at the laboratory. Clinical details were kept separately at the RLBUHT NHS hospital site.

In addition to histological samples taken for routine patient care, up to twelve small and large bowel biopsies were taken, with informed written consent, from each patient recruited to the aphthous ulcer trial, with standard single use sterile endoscopic forceps used. Biopsies were taken from areas of aphthoid ulceration, lymphoid follicles, Peyer's patches, and ileal tissue, as well as colonic samples including normal mucosa. Patients had standard bowel preparation with Moviprep (Norgine). The biopsies were put individually into 1.5 ml tubes containing 500 µl of sterile saline, and these samples were placed on ice and taken back to the laboratory for qPCR analysis.

In the plantain trial patients did not receive bowel preparation and the four sigmoid colon samples, taken at between 15 and 25 cm from the anal margin, were similarly placed in sterile saline on ice, and taken for DNA extraction and qPCR (see chapter seven).

5.11.2 Removal of the adherent mucus layer to evaluate mucus associated bacteria

Samples used for qPCR from each patient were placed in 500 µl 0.016% (w/v) dithiothreitol (DTT) made up in sterile saline in 1.5 ml tubes (as per (232)). These

were rotated for 15 min to remove the mucus layer. The supernatant was removed for each sample separately and then these biopsy samples were then washed three times by transferring them into consecutive 1.5 ml tubes containing 500 µl of sterile saline, by pipette suction. Each of these washings was pooled with the original supernatant from each biopsy and these were then centrifuged at 10,000 rpm (7830 g) for 10 min to produce a bacterial pellet, for assessment of the mucus-associated bacteria by qPCR.

The residual DTT-treated biopsies and bacterial pellets were placed directly in the -80°C freezer for storage prior to genomic DNA extraction.

5.11.3 DNA extraction from human biopsies and bacterial pellets

Human biopsies and their associated pellets had human and bacterial DNA extracted from them as described in section 5.3.2.

CHAPTER SIX : THE DEVELOPMENT OF A Q-PCR ASSAY FOR QUANTIFICATION OF BACTERIA IN ENDOSCOPIC INTESTINAL MUCOSAL SAMPLES

6.1 Aim:

To develop a quantitative polymerase chain reaction (qPCR) assay for quantification of mucosal bacteria with sufficient sensitivity and reproducibility for use on endoscopic biopsy samples obtained in clinical trials in CD.

6.2 Introduction

6.2.1 Direct quantification of bacteria by PCR amplification

To develop a method for direct quantification of bacteria by PCR meant a number of issues had to be addressed. These issues were sensitivity, use of an appropriate denominator, choice of an appropriate candidate gene for bacterial quantification and choice of an appropriate quantification method.

6.2.1.1 Determining the required sensitivity

It was anticipated that quantifying bacterial numbers from endoscopic biopsies by qPCR might be difficult. Even when DNA is optimally extracted from biopsy tissue, it is known that bacterial DNA template numbers from intestinal biopsy tissue can be expected to be low; one might expect in the order of 10^6 total bacteria/mg intestinal tissue, and from work in this thesis, for DTT-treated samples, average biopsy wet mass was found to be between 8-115 mg at the extremes of sampling for both CD and control samples (n=54, mean mass 48.4 ± 3.8 mg SEM (308), meaning an expectation of 4.84×10^7 aerobic bacteria per biopsy. Previous work from our own laboratory in Liverpool yielded results in the order of mucosa-associated bacteria (0-1000 CFU per biopsy, median >100), mucus-associated (0-3000 CFU per biopsy, median >10), and intracellular (0-500 CFU per biopsy, median >1). These figures include all aerobic bacterial species cultured and not simply *E. coli*. These *E. coli* accounted for about 50 % of cultures of mucosa-associated bacteria after mucus removal (232).

6.2.1.2 Standardisation and choice of appropriate denominator

Standard curves can be generated by taking serial bacterial suspension dilutions and in parallel (i) plating the bacteria out on standard LB agar and (ii) extracting DNA from the same suspensions and then analysing them in a qPCR reaction. The results may be compared by correlating colony forming units (CFU) against PCR crossing point (Cp). Crossing point refers to the earliest cycle number at which the accumulation of

fluorescence from a qPCR reaction rises above background fluorescence level. It is inversely correlated with the gene or DNA template quantity in the qPCR reaction.

Alternative approaches to producing a standard curve include expressing bacterial numbers per tissue cells following DNA extraction, or using tissue DNA as denominator, with qPCR used for both bacterial and epithelial cell quantification (218), or as number of bacterial genomes per wet weight of biopsy tissue (306).

In order to produce standard curves relating gene copies to bacterial numbers, either 'bulk' quantities of candidate bacterial chromosomal DNA can be extracted and quantified spectrophotometrically (306), or the gene of interest can first be amplified and cloned into a plasmid vector. These plasmid-amplicon products can then be incorporated into chemically or electrocompetent cells and used for generation of a large amount of plasmid-amplicon DNA, quantified by sophisticated spectrophotometry such as use of the Nanodrop™. Using this DNA, the target sequences (genes) of interest can be quantified in terms of copy number and used to generate a calibration curve (309;310). This approach allows precise calculation of the number of target genes present in standard plasmid DNA template quantities and it was ultimately used in this work.

Each method makes certain assumptions and has limits of sensitivity and precision. Semi-quantitative assessment of bacterial numbers may also be achieved using fluorescence in situ hybridization (FISH) (218;311), but this method is perhaps better used to show distribution of bacteria in tissue and was not used here.

6.2.1.3 Use of appropriate candidate genes and the relevance of viability in quantification of bacterial numbers

The *uidA* gene has been used previously in studies for quantification of *E. coli* (218;226). It is specific for *E.coli* (304) and was used here. The *uidA* gene, encoding the β -glucuronidase enzyme, is assumed to be present as a single copy per *E. coli* genome (218),(312) and is certainly known to be present in a single copy in the *E. coli* K-12 genome (NCBI database). The possibility of more than one copy in different *E. coli* strains has been suggested, though no definitive evidence has been presented (313).

A direct correlation between CFU and PCR products is not always found, as an amplified piece of DNA does not necessarily correspond to a viable micro-organism upon agar plating. In some studies this has produced an over-estimate by a log factor of 1.5 (312). Separation of live from dead bacteria quantified by PCR can be achieved staining with ethidium monoazide. This reagent will not pass through the cell wall of live bacteria (312) but intercalates into dead bacterial cells, and inhibits dead cell DNA amplification. This technique was not employed in this work, as presence of bacteria, alive or dead, was of interest in this research, as we were interested in the quantification of bacteria, and if *E.coli* were found attached to mucosa or in the associated mucus, they can be assumed to be alive at some point to get to these locations.

6.2.2 Minimum statistical requirements required by the qPCR assay

There is considerable variation in the quality of statistical analysis and presentation of qPCR data in the literature, and consequently ideal minimum standards have been proposed (see table 6.1 below). These criteria are addressed throughout this work in chapters five, six, and seven. It is necessary to produce sufficient experimental detail of qPCR setup to allow replication of assays, these standards are discussed in detail in (314). In addition, to these standards, a further specification of sensitivity for the assays was stipulated in this work, namely that quantifying bacteria in the order of 1000 would be the lower limit of detection for the assay. Good reproducibility (low coefficient of variability) is also desirable. I will now define and discuss the relevant terms:

6.2.2.1 Efficiency of a PCR reaction

The slope of a standard curve (C_p plotted against \log_{10} gene copy number) describes the kinetics of a PCR reaction and is the efficiency of the amplification reaction. It indicates how much the target nucleic acid can be expected to increase with each cycle. A perfect amplification reaction has an efficiency of 2 (which is the LightCycler® machine equivalent of 100%), when the target nucleic acid is doubled in every amplification cycle.

$$T_n = T_0 \times 2^n$$

Where T_n is the number of target molecules at cycle n , n is the cycle number, and T_0 is the number of target molecules present at the start of the reaction. This relationship is true for a perfect amplification reaction during the log-linear phase.

However, since real life PCR reactions are not perfect, non-specific amplification of other nucleic acid sequences can make the efficiency above or below 100%, and therefore the efficiency (E) is calculated by:

$$E = 10^{-1/\text{slope}}$$

where slope is the standard curve gradient. In essence therefore the amplification efficiency is related to the linear regression line calculated from the calibration curves for standard DNA templates (315). The ‘acceptable minimum efficiency’ range for an assay is within 90-110% (expressed as 1.8-2.2 on the LightCycler® machine) The source for these equations is the following web address (Roche):

http://icob.sinica.edu.tw/pubweb/Core%20Facilities/Data/R401-core/LightCycler480%20II_Manual_V1.5.pdf : section 4.2.2, p162-163.

6.2.2.2 Correlation coefficient (r^2) for quantification assays

This is a measure of association between the dependent and independent variables (316). In these experiments the dependent variable is the PCR C_p (defined in section 6.2.1.2 - inversely correlated with quantity of template) and the independent variable is \log_{10} quantity of template, be that CFU, genomes, or genes. In other words, r^2 defines the proportion of sum squared points which are accounted for by the linear

regression of the dependent and independent variables. The acceptable minimum requirements for correlation coefficient is a calculated value >0.95 .

6.2.2.3 Error of a PCR reaction

The Error rate is similar to correlation coefficient but is a more accurate calculation of “variance”; the *Error* value (mean squared error of the single data points fit to the regression line), is a measure of the accuracy of the quantification result based on the standard curve (an acceptable value should be < 0.2)" (from the LC480® user manual, weblink as above, 6.2.2.1). In essence it is an averaged measure of how far the standard points are off the regression line.

6.2.2.4 Sensitivity or limit of detection in PCR

In real-time PCR, sensitivity refers to the minimum number of copies in a sample that can be measured accurately within an assay. It is usually expressed as the limit of detection (LOD), which is the concentration that can be detected within a ‘reasonable certainty’ (95% usually). 3 copies per PCR is the minimum theoretically possible LOD, assuming a Poisson distribution, a 95% chance of including at least one copy in the PCR, and single copy detection. Results which specify ‘zero’ for quantities are also meaningless; firstly it is more appropriate to state ‘not detectable’, and secondly, PCR results are usually expressed as a logarithm of a quantity (due to the nature of the PCR reaction), and a logarithm of zero is unquantifiable (314). When template concentration is zero the crossing point is also undefined (due to the logarithmic nature of qPCR) which also makes LOD estimates complicated. Appropriate

determination and expression of LOD as an issue in qPCR is being explored (314;317), due to these ambiguities. For these experiments in view of the known difficulties of detection of low copy numbers, (314;317), and the estimated requirement for the assay based on CFU seen in previous work (3000, see (232)), attempts were made to try and match this magnitude with an LOD of 1000 bacteria.

6.2.2.5 Reproducibility of PCR assays or coefficient of variability

This describes long term precision or inter-assay variance in the results between runs and is typically expressed as the coefficient of variability (CV); this is the SD for aliquot data expressed as percent of mean (314). My target for this reproducibility, in line with a recent PCR publication, was <20%, and ideally <10% (318).

Table 6.1 Ideal minimum qPCR standards checklist. Checklist for authors' of MIQE précis (from (319)), detailing information about individual parameters associated with each step of the RT-qPCR workflow.

NAC = non amplification controls, **NTC** = non template control, **RT** = real time.

Sample/Template	Details
Source	If cancer, was biopsy screened for adjacent normal tissue?
Method of preservation	Liquid N ₂ /RNAlater/formalin
Storage time (if appropriate)	If using samples >6 months old
Handling	fresh/frozen/formalin
Extraction method	TriZol/columns
RNA: DNA-free	Intron-spanning primers/no RT control
Concentration	Nanodrop/ribogreen/microfluidics
RNA: integrity	Microfluidics/3':5' assay
Inhibition-free	Method of testing
Assay optimisation/validation	
Accession number	RefSeq XX_1234567
Amplicon details	exon location, amplicon size
Primer sequence	even if previously published
Probe sequence*	identify LNA or other substitutions
<i>In silico</i>	BLAST/Primer-BLAST/m-fold
Empirical	primer concentration/annealing temperature
Priming conditions	oligo-dT/random/comboination/target-specific
PCR efficiency	Dilution curve
Linear dynamic range	spanning unknown targets
Limits of detection	LOD detection/accurate quantification
Intra-assay variation	copy numbers not C _q
RT/PCR	
Protocols	detailed description, concentrations, volumes
Reagents	supplier, Lot number
Duplicate RT	▲ C _q
NTC	C _q & melt curves
NAC	▲ C _q beginning:end of qPCR
Positive control	inter-run calibrators
Data analysis	
Specialist software	e.g., QBAsePlus
Statistical justification	e.g., biological replicates
Transparent, validated normalization	e.g., GeNorm summary

6.3 Detailed aims

1) To develop suitable qPCR assays for the detection of mucosa-associated bacteria in human biopsies with a sensitivity of approximately 1000 bacteria per 5µl of template DNA extracted from human intestinal biopsies. Reviewing the literature expected *E. coli* quantities in intestinal biopsy tissue vary depending on the method used for quantification, sometimes quite widely if PCR techniques and those involving CFU are compared. There is a paucity of studies directly comparing *E.coli* numbers using a single standardised PCR method, and therefore the quantity of bacteria expected in different tissues quantified using this method was unpredictable. In one study *E.coli* were quantified by qPCR (218) and results were expressed as *uidA* genes per million (18S rRNA genomes) human cells. Assuming one copy of the *uidA* gene per *E.coli* bacterium, and extrapolating this study's figures for an average sized biopsy found in our work (18S rRNA results in chapter seven), to correct for size differences, one could expect 469–4690 *E.coli* from DNA extracted from ileal tissue, with only 12 *E.coli* per biopsy of colonic tissue, and 7 *E.coli* per normal biopsy, but these figures were only based on one study expressing results in one way, and had to be regarded circumspectively (218).

From previous work at this laboratory which examined all (aerobically) cultured microbiota from colonic biopsy tissue, using plated colony counts of control and CD samples, quantities expected per biopsy could be anywhere between 0 and 3000 total aerobic bacteria per biopsy, with the higher figures seen in CD colonic tissue (232). Clearly, the technique used for quantification by CFU assessment is different from quantification by qPCR of DNA extracted from microbiota. The huge difference between techniques is emphasised by the disparity seen in *E.coli* numbers

found in each study in colonic tissue. This may reflect technique, patient disease or inherent variability. It does emphasise the need for a clear denominator in quantification studies.

In an additional refinement of the main chapter aim, the bacterial quantification assay should have PCR efficiency between 90-110% and a correlation coefficient of at least 0.95, with reproducibility demonstrating a coefficient of variation between assays of <20%.

2) To construct a standard curve for qPCR of human 18S rRNA genomes to utilise as a denominator for standardisation of bacterial quantity per mucosal biopsy size for use in CD clinical trials. The qPCR technique used would have to satisfy the same minimum statistical requirements as specified above (aim 1) for the bacterial assay.

6.4 Methods and results

Detailed methods are in chapter five, the relevant sections are specified throughout the text below.

6.4.1 Screening with endpoint PCR to detect *uidA* genes

Previous authors had used the *uidA* gene in quantification assays for *E. coli* (218;312;313) on the assumption, proven for some UPEC and *E. coli* K-12, that there is a single copy of the gene per bacterium. It was important to demonstrate the

presence of this candidate gene in our library of CD *E. coli* isolates as a starting point, as well as to show a negative PCR in non-*E. coli* bacteria. Searches of the NCBI nucleotide database using *uidA* primers F1 R1 (see table 5.1, chapter 5) confirmed their presence in *E. coli* strains and *Shigella spp.* but not in any other species. *Shigella* are considered by some to belong to the same species as *E. coli* (244).

DNA was extracted from a selection of six colonic and two ileal CD *E. coli* ‘library’ strains (origin of strains discussed in section 5.2.1). Endpoint PCR for the *uidA* gene was then carried out as described in 5.4.

PCR amplification and DNA agarose gel electrophoresis (as described in section 5.4.1) demonstrated the presence of the *uidA* gene in a representative sample of the DNA of our colonic CD *E. coli* isolates from Liverpool patients, as well as ileal strains LF-10 and LF-82 from French patients, whereas the negative bacterial control *Klebsiella pneumonia* was PCR-negative (shown in figures 6.1 and 6.2).

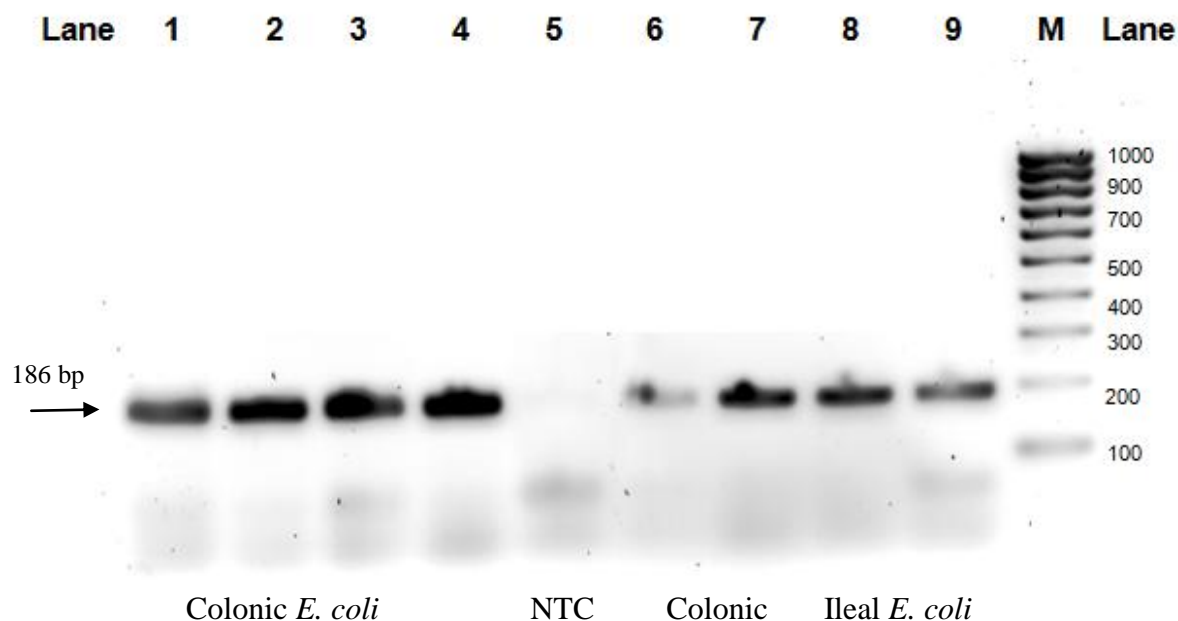


Figure 6.1 Screening of the ileo-colonic CD isolates for presence of the *uidA* gene.

The Appearance after CD *E. coli* isolates have undergone DNA extraction, endpoint PCR, and electrophoresis using a 2% (w/v) DNA agarose gel in TAE.

Lane 1-4, and 6-7 : Colonic isolates HM670, HM615, HM605, HM580, HM154, and HM95

Lane 5: PCR water (NTC). Lane 8 and 9: ileal isolates LF-82 and LF-10

M: Marker lane, DNA Hyperladder IV 100-1000bp.

This image demonstrates that the *uidA* gene is present in representative *E. coli* isolates from our CD library (232), as well as some ileal isolates (233).

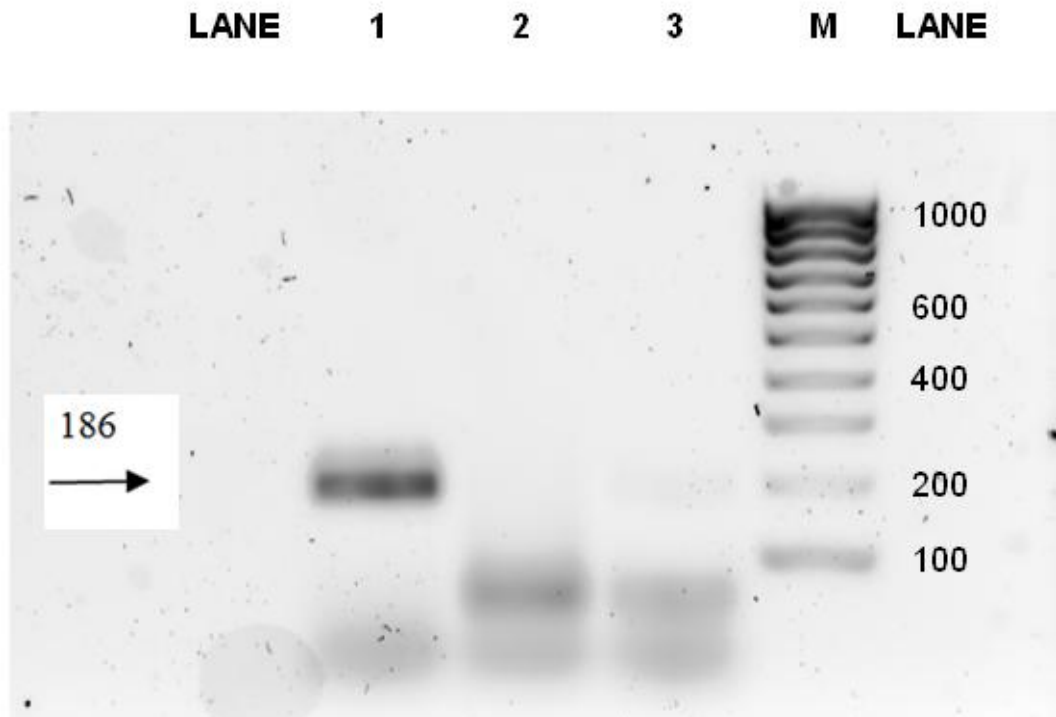


Figure 6.2 The bacterial control *Klebsiella pneumonia* (HM534) is PCR-negative for the *uidA* gene. Amplicons were analysed using a 2% (w/v) agarose gel in TAE.

Lane 1: *E. coli* HM605, Lane 2: PCR water, and Lane 3: *Klebsiella pneumonia* HM534. M: Marker lane, DNA Hyperladder IV 100-1000bp.

As the specificity of the *uidA* gene for quantifying *E.coli* in CD patients had been supported, the optimum standardisation method was then evaluated.

6.4.2 Methods for generating a standard curve for *E .coli* quantification

Four approaches were compared for quantification of bacteria, reflecting previously attempted strategies in the literature.

Method 1: Crude extraction of genomic DNA from serial dilutions of bacteria with this DNA used in qPCR.

Method 2: Extraction of DNA from an initial bacterial suspension using a commercial DNA extraction kit, with the extracted DNA and initial bacterial suspension diluted in parallel. For both methods one and two the bacterial suspensions were also agar plated and CFU enumerated by overnight culture. Method 2 involves higher purity DNA extraction compared to method 1.

Method 3: Extraction of genomic DNA from an initial bacterial suspension as for method two but with dilution of this DNA, coupled with the use of spectrophotometry and published bacterial genome sizes to calculate the bacterial numbers present in DNA dilutions used.

Method 4: Insertion of an amplicon from the *uidA* gene of interest in single copy into a plasmid vector. This was then followed by plasmid transformation of competent cells, extraction of plasmid DNA from these cells and subsequent quantification of plasmid copies with spectrophotometry. Methods 3 and 4 do not involve agar plating.

6.4.2.1 Method One. Production of a standard curve for *E. coli* quantification using bacterial CFU and PCR Cp

The representative CD isolate *E. coli* HM605, which is an adhesive/invasive haemagglutinin-positive isolate that has been used for *in-vitro* work previously (242), was used for standardisation purposes. An *E. coli* suspension (see section 5.2.4) was serially diluted 4 times with PBS, each one in ten. The most dilute suspension was then further diluted 6 times, each one in two. Volumes were removed from each subsequent suspension and used for both qPCR (100µl) and agar plating (200µl) in parallel (see sections 5.2.4, 5.7.1, and 5.7.2). This technique was based on previous work (218) and included the expected CFU range from CD and control tissue. Optical density (OD) was found to be an unhelpful measure in this range and was not utilised.

6.4.2.1.1 *uidA* qPCR primer optimisation experiments

Conditions for the *uidA* primers F2 R2 were optimised (5.6.1) before they were used to define the qPCR Cp of DNA extracted from bacterial suspensions. Primer concentrations of 300 and 900 nM were effective, but with a lower signal seen with both forward and reverse primer concentrations of 300 nM (figures 6.3 and 6.4). Forward and reverse primer concentrations of 900 nM, allowing the best signal and easy co-dilution, with a probe concentration of 200 nM were chosen as the optimal concentration for use in further experiments evaluating *E. coli* DNA templates. The final optimised annealing temperature was 60°C.

6.4.2.1.2 Determining qPCR crossing point for the *E. coli* suspensions

DNA was extracted from *E. coli* bacterial suspensions and qPCR was performed as per sections 5.3.1 and 5.7.1 - 5.7.2 using the optimised *uidA* primers F2 R2 (see chapter 5, table 5.1 and results figure 6.5). The LC480® software enabled each qPCR reaction to be ascribed its corresponding CFU value from the parallel agar plating (see section 5.2.4), and generation of a standard curve (figure 6.6). None of the qPCR fluorescence curves reached the plateau phase, suggesting that a process increasing purity and yield of *E. coli* DNA extracted was required to improve qPCR, and standardisation.

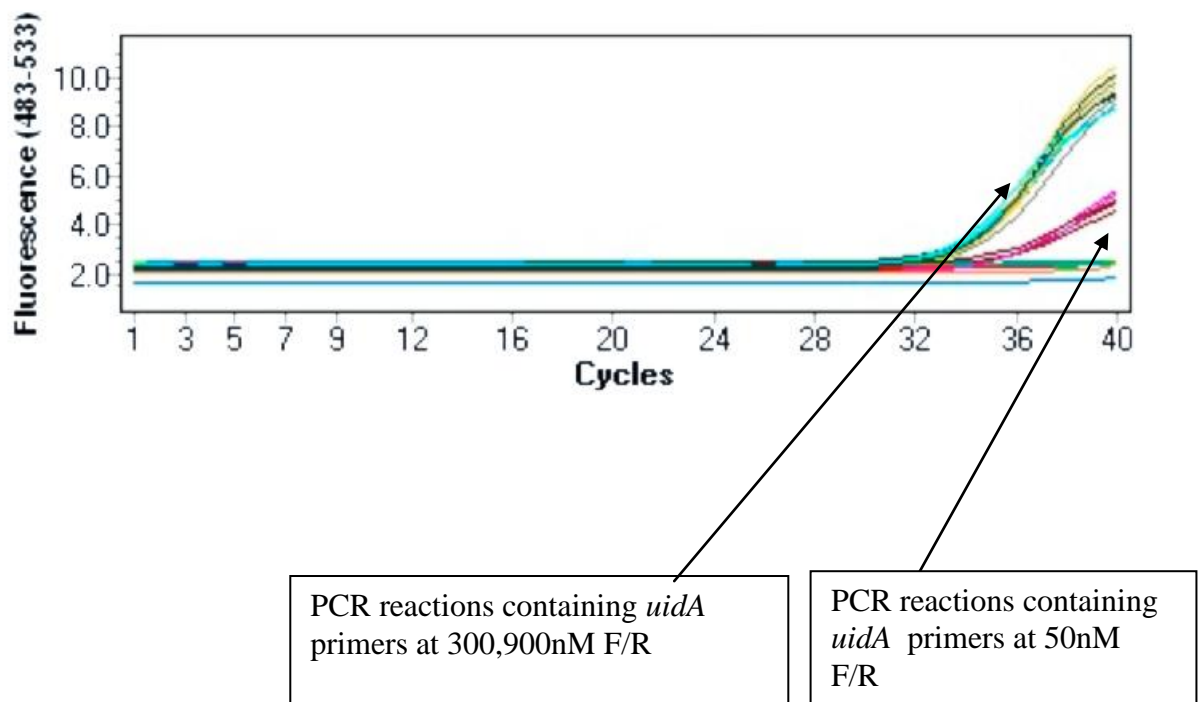


Figure 6.3 Primer optimisation experiment using an acquisition temperature of 60°C showing poor signal from 50nM primers and optimal signal with 300 and 900nM concentration primers. Template was *E. coli* HM605, 10^7 CFU/ml.

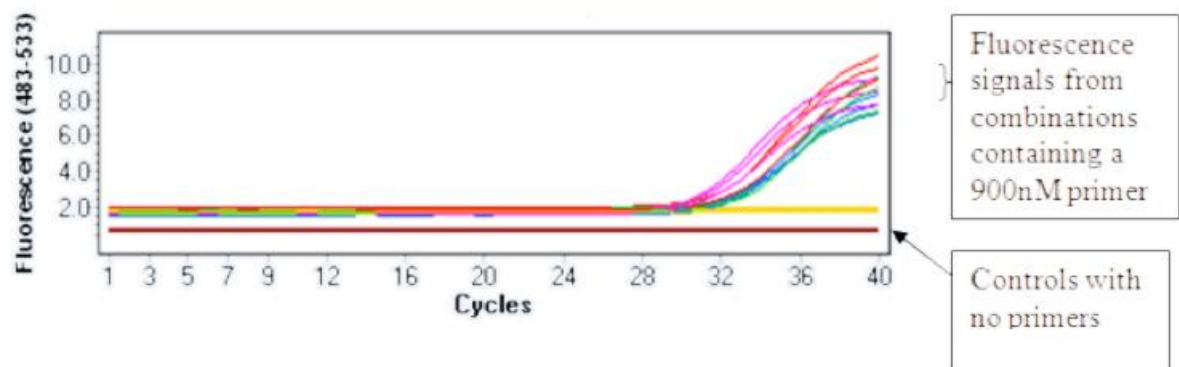


Figure 6.4 Final primer optimisation experiment. Forward and reverse primer concentrations of 300 and 900nM were evaluated in the following combinations: 300-300,300-900,900-300 and 900-900). A suspension of 10^7 CFU/ml HM605 *E. coli* was used as template. qPCR of *uidA* genes shows similar signals for all primer concentrations used, with a lower signal for forward-reverse primer concentrations of 300-300 nM (blue curves). The primer-less controls show background signal only

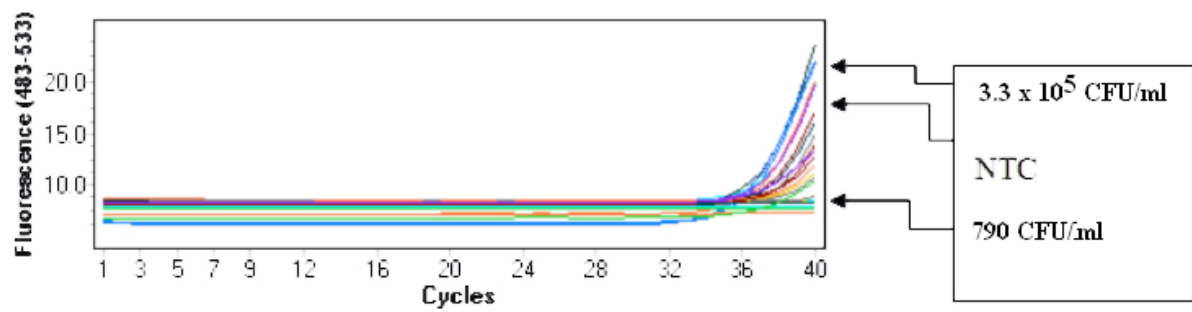


Figure 6.5 qPCR evaluation of DNA extracted crudely from bacterial suspensions. qPCR primer concentrations were 900 nM, with probe 200 nM. DNA was extracted by boiling and centrifugation from *E. coli* bacterial suspensions that on agar plating were equivalent to between 790 and 3.3×10^5 CFU/ml. The fluorescence curves obtained by qPCR are shown. NTC (red curve) has a positive signal and most of these curves have $C_p > 35$.

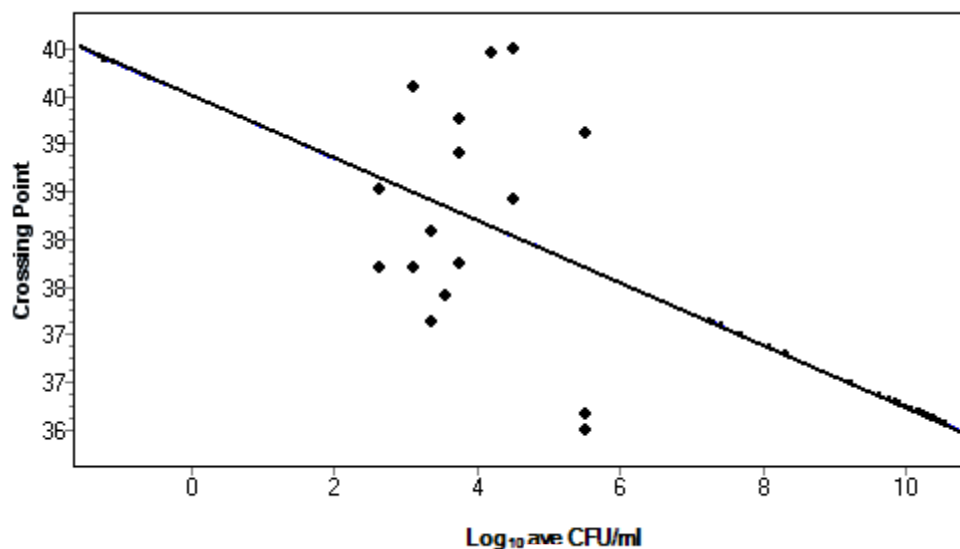


Figure 6.6 The standard curve obtained using method one. The standard curve is generated by *uidA* qPCR of DNA extracted from *E. coli* bacterial suspensions. The y-axis shows Cp, (the average of triplicates), for the DNA template from each bacterial suspension plotted against the log of the average corresponding CFU numbers (x-axis) derived from each suspension by agar plating (efficiency 59.1% with error 0.383).

6.4.2.1.3 Conclusion for method one

The standard curve produced had an efficiency of 59.1% with an error of 0.383. The LOD of the assay was at least 3.3×10^5 CFU. These were clearly not within the defined acceptable parameters for the assay.

6.4.2.2 Standardisation method two: use of the Qiagen DNA extraction kit prior to qPCR quantification of *E.coli*

The two differences in method two compared to method one were, firstly, that the initial bacterial suspension of *E. coli* HM605 prepared at 1×10^9 CFU/ml had DNA extraction performed using the DNA minikit, (section 5.3.2) to increase yield and purity. This had been used in previous standardisation work to extract DNA from *H. pylori* (306).

Secondly, the DNA extracted from the initial suspension was diluted, rather than extracting DNA after each serial dilution of the bacterial suspension. Bacterial CFU were again assessed by agar plating as described in 5.2.4, with CFU standardised per ml. DNA was used in qPCR reactions as described in 5.7.1 – 5.7.2.

6.4.2.2.1 Results for PCR quantification of mucosal bacteria - method two

Using method 2, excellent correlation was seen between crossing point and CFU (see figures 6.7 and 6.9). The coefficient of variation for the CFU enumeration process was similar and acceptable for both intra- and inter-experimental comparisons (Table 6.2). The fluorescence curves generated by qPCR of the DNA extracted and diluted from the initial *E. coli* HM605 suspension (1×10^9 CFU/ml) are shown in figure 6.8 and the corresponding C_p in figure 6.9. The fluorescence curves representing a range from 7.9×10^2 to 2.2×10^5 CFU/ml are very close together and do not reach the plateau phase, but do show increasing C_p value with increasing dilution. The black fluorescence curve, which represents signal in NTC (0 CFU/ml)

has approximately the same signal as qPCR of DNA template extracted from a suspension of 2.2×10^5 CFU/ml.

The implication from these curves was that more concentrated DNA extraction or template availability from the bacterial suspensions in the range of interest would be required to achieve adequate sensitivity as the dilution corresponding to the highest bacterial CFU numbers seen in previous CD biopsy studies (in the order of 3000 CFU per biopsy (232)) did not reach plateau.

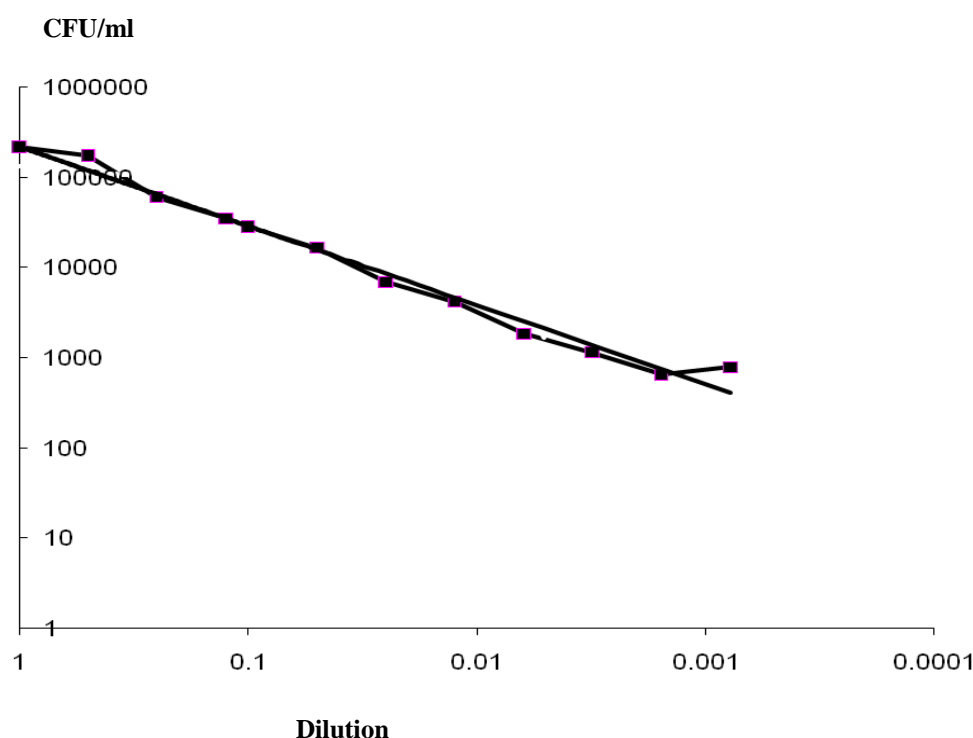


Figure 6.7 Demonstration of a linear relationship between CFU and dilution of bacterial suspension. Both axes are plotted logarithmically. Standardised CFU/ml versus bacterial suspension dilution, where each dilution is expressed as a value relative to the original (1) with 1/2 dilution = 0.5, 1/10 dilution = 0.1 etc) This gives a straight line (equation $y=21851x^{0.878}$) with correlation coefficient $R^2 = 0.983$.

Table 6.2 Coefficients of variation for the CFU plating component of standardisation methods 1 and 2. Co-efficients of variation (CV) for the colony counting technique used in methods 1 and 2. CV is equal to the standard deviation of a set of readings divided by the mean of that set of readings multiplied by 100.

Analysis	CV (%)
Intra-method 1 (n 12)	18.28
Intra- method 2 (n 12)	18.12
Inter-experiment (N 2 series)	18.2

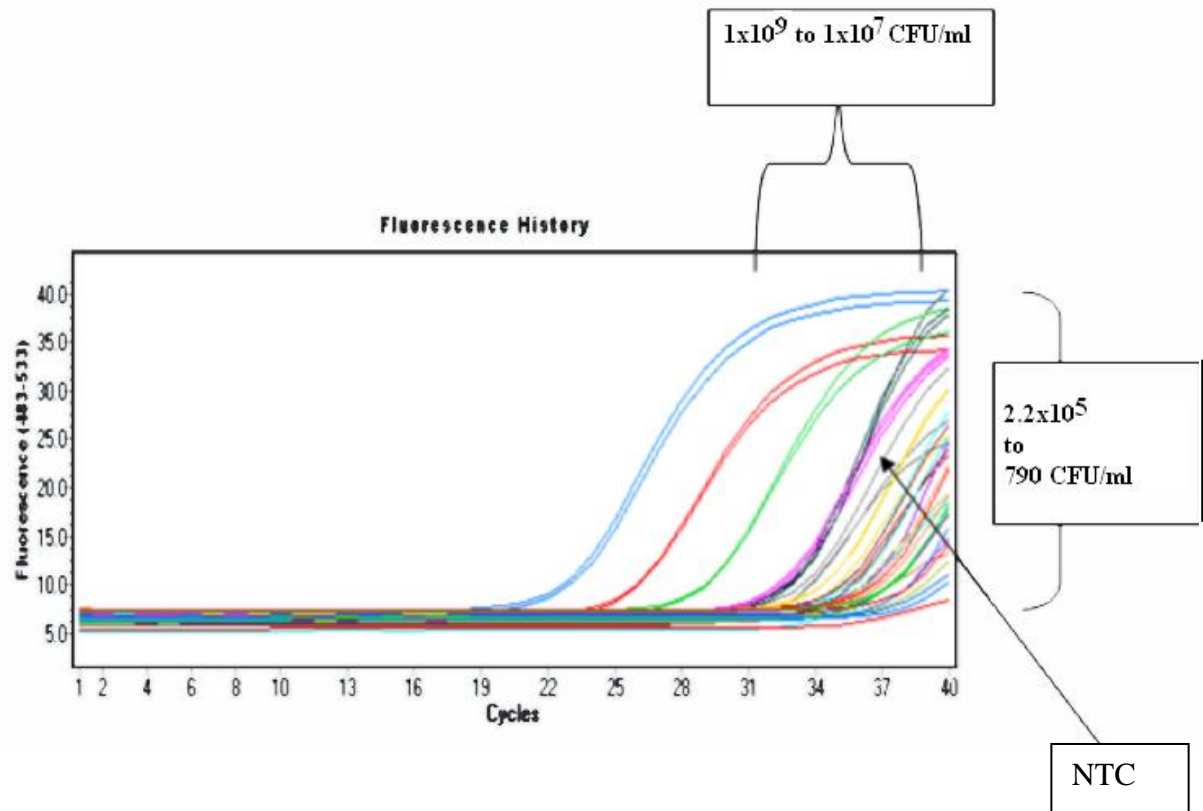


Figure 6.8 Fluorescence curves generated by qPCR of DNA extracted from *E. coli* bacterial suspensions using the Qiagen tissue minikit (method 2). The blue, red, and green curves represent standard dilutions of *E. coli* DNA corresponding to bacterial suspensions of approximately 10^9 , 10^8 , 10^7 CFU/ml, (not plated) respectively. The other fluorescence curves moving from left to right are in the area of interest 2.2×10^5 to 790 CFU/ml, (or purple to orange). The black fluorescence curve represents signal in the NTC.

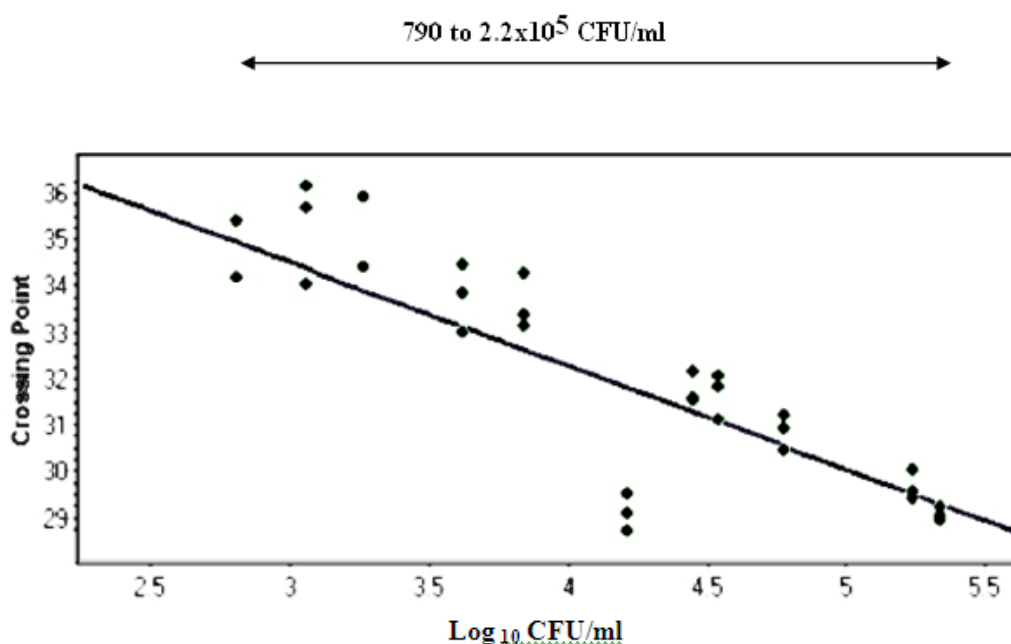


Figure 6.9 Standard curve generated by method 2 - A standard curve was produced using the bacterial CFU suspensions 2.2×10^5 to 790 CFU/ml, which had an efficiency of 2.814 (141%), and error of 0.298. The standard curve is generated by fit points analysis (all settings automated) using the LC480® software. Cp for each corresponding DNA template is plotted against log of standardised CFU/ml for bacterial suspensions.

6.4.2.2.2 Conclusions regarding PCR bacterial quantification method two

Although the standard curve generated by this method was greatly improved relative to method one, with a PCR efficiency of 2.814 (141%), and an error of 0.298, and LOD of at least 2.2×10^5 (figure 6.9), an alternative, more sensitive method for bacterial DNA quantification was needed.

6.4.2.3 Bacterial quantification method three – production of a standard curve following calculation of bacterial quantities using published genome size and spectrophotometry of bacterial DNA

In order to improve assay sensitivity and precision, another technique for absolute quantification of bacterial numbers, which had been used previously in the literature, was attempted. This method utilises absolute genome numbers for bacteria (306), calculated from published bacterial genome size and accurate quantification of extracted genomic bacterial DNA quantity using the Nanodrop™ machine. Agar plating was not involved with this method and a DNA kit optimised for extracting bacterial DNA was used to enhance initial DNA template quantity. This was the Promega Wizard® Genomic DNA Purification Kit (A1125) described in section 5.3.3. This addressed the previous problems with sensitivity of the standardisation methods, as well as improving DNA extraction from bacteria and explored the effect of removing CFU quantification from the standardisation process.

6.4.2.3.1 Quantification of bacterial genome copy numbers

After DNA was extracted from multiple *E. coli* suspensions, these were quantified, and the sample with the highest purity and greatest concentration was used as template for the standard curve (385 ng/μl and Absorbance at 260 nm/280 nm ratio 1.84).

E. coli genome numbers were calculated based on first principles:

1) *E. coli* genome size = 4.7-5.1 Mbp, assumed to be 5.0 Mbp, and relative molecular mass (R.M.M.) of a DNA bp = 660 relative mass units

Therefore *E. coli* genome RMM = $660 \times 5 \times 10^6 = 3.3 \times 10^9$

2) From formula for mass, RMM, and moles:

Mass (g)/ RMM = number of moles

Moles = 3.85×10^{-7} g (the best DNA extraction quantity per μl found above) / 3.3×10^9
= 1.17×10^{-16} moles

3) Avogadro's number N_A = number of molecules in a mole = 6.022×10^{23}

Therefore molecules of *E. coli* genome present per μl DNA = $6.022 \times 10^{23} \times 1.17 \times 10^{-16}$
moles = 7.05×10^7 genomes per μl .

Therefore in the $5\mu\text{l}$ templates used in the PCRs this DNA concentration gives
 3.53×10^8 genomes.

6.4.2.3.2 Production of a standard curve for *E. coli* quantification by qPCR of *E. coli* linear genomic DNA vs PCR Cp

The genomic *E. coli* DNA was serially one in ten diluted using PCR grade sterile water to produce a range of *E. coli* genome numbers between 3.53 and 3.53×10^8 , or DNA quantities between $3.85 \text{ fg } \mu\text{l}^{-1}$ and $385 \text{ ng } \mu\text{l}^{-1}$. DNA template was used at $5\mu\text{l}$

volumes in qPCR assays with PCR water (Roche) used as NTC. qPCR was performed on these *E. coli* DNA samples using SYBR green technology and *uidA* primers F1 R1 (see table 5.1, chapter 5 and section 5.7.3). In addition to the *E. coli* genomic DNA, crudely extracted DNA (section 5.3.1) from bacteria which do not contain the *uidA* gene were also used as negative controls, namely *Enterococcus faecalis*, *Citrobacter Rodentium*, and *Klebsiella pneumonia*.

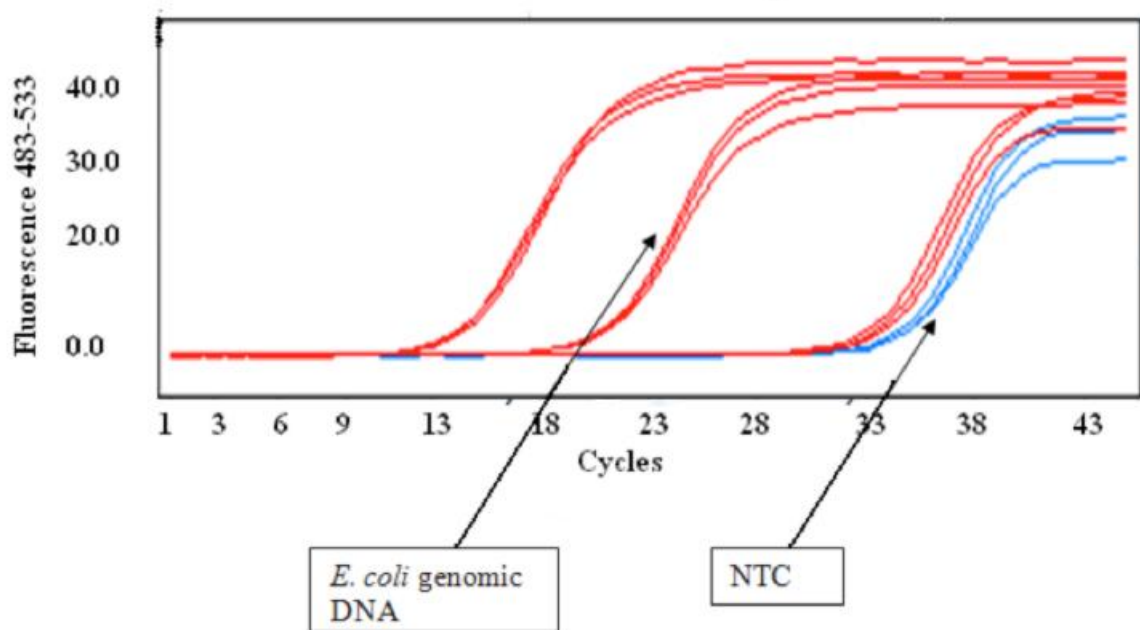
Melt curve analysis was used with SYBR green qPCR to confirm the specificity of products.

6.4.2.3.3 Results for bacterial quantification method three

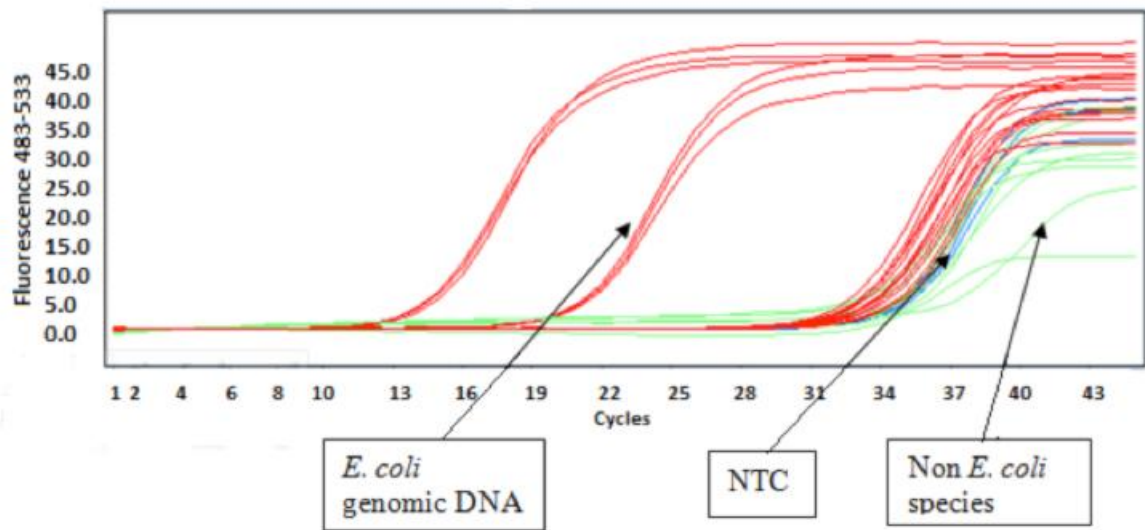
qPCR fluorescence curve results are shown in figure 6.10 with the resultant standard curve in figure 6.11 and melt curve analysis shown in figure 6.12.

Use of the *uidA* primers F1 R1 for qPCR of genomic DNA allowed clear differentiation of *E.coli* numbers from NTC, with a LOD of 3530 genomes or a DNA concentration of $3.85 \text{ pg } \mu\text{l}^{-1}$ (figure 6.10A). *Enterococcus faecalis* (HM65), *Citrobacter Rodentium* (HM481), and *Klebsiella pneumonia* (HM 510) species had similar fluorescence signal to NTC (included in **B**), confirming that these species do not contain the *uidA* gene. Figure 6.10B shows that PCR templates containing 3.53 - 3530 *E. coli* bacterial genome equivalents produced the same signal as NTC, suggesting that using *uidA* qPCR and genomic bacterial DNA, the limits of bacterial detection, or sensitivity, were being reached. Figure 6.11 demonstrates the standard curve produced by these genome standards used in qPCR.

The melt curve (figure 6.12) demonstrates a prominent peak for a unique product with a small secondary peak which encompasses a melt curve product produced by templates with Cp greater than or equal to NTC when used in qPCR. This may represent small amounts of *E. coli*, or non-specific PCR products, but quantitatively it is unlikely to have any significance.



A



B

Figure 6.10 qPCR fluorescence curves produced by *E. coli* genomes and negative bacterial controls used in quantification method three.

uidA qPCR of *E.coli* genomes (red curves represent dilutions of *E. coli* genomic DNA, not all templates are shown for clarity). The LOD indicated represents 3530 genomes or a DNA quantity of $3.85 \text{ pg } \mu\text{l}^{-1}$ **A**, with *Enterococcus faecalis*, (HM65) *Citrobacter Rodentium*, (HM481) and *Klebsiella pneumonia* (HM 510) species having similar fluorescence signal to NTC (green curves included in **B**). Blue represents NTC (PCR water) in both **A** and **B**.

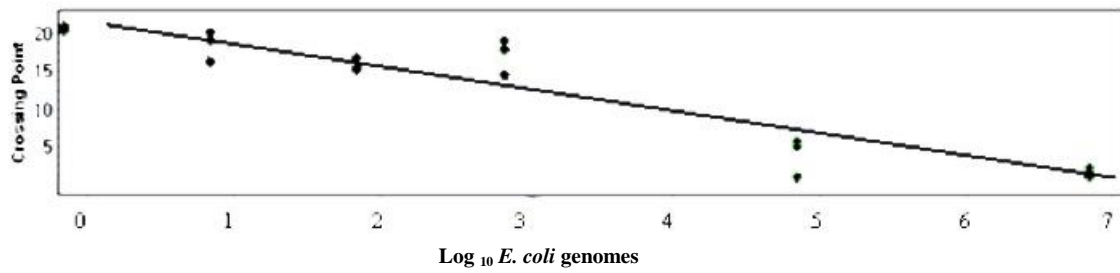


Figure 6.11 The standard curve generated using genomic *E. coli* DNA and the nanodrop to quantify bacterial numbers. PCR efficiency is 2.207 (110.35%) with an error of 0.896.

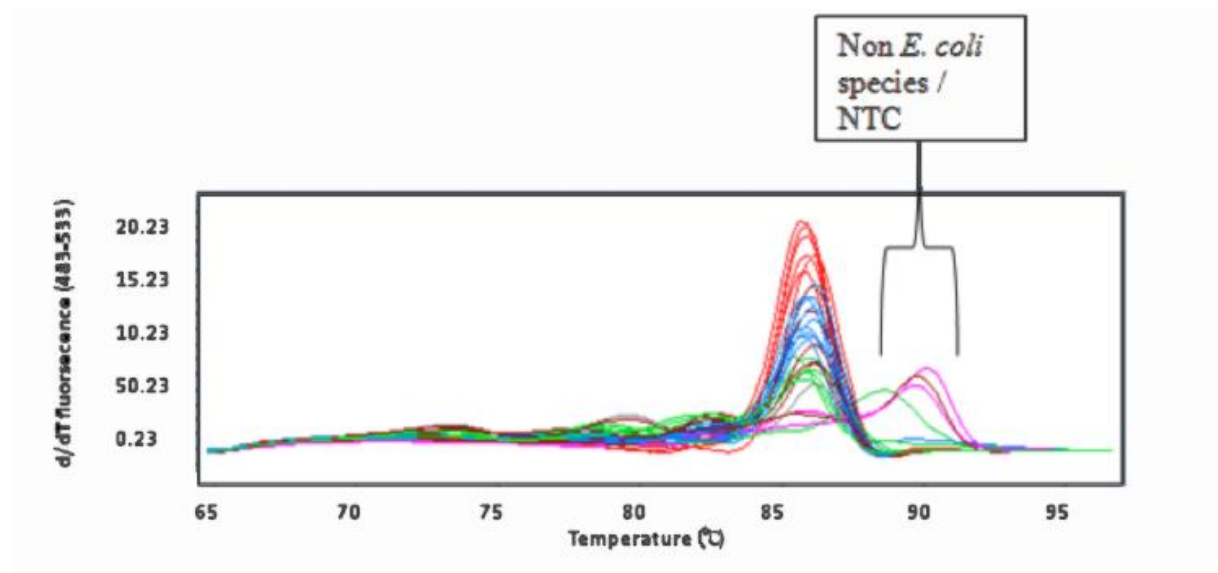


Figure 6.12 Melt Curve demonstrating uniqueness of *uidA* amplicon product from genomic *E. coli* DNA. The second peak represents NTC and non *E. coli* bacterial species.

6.4.2.3.4 Conclusion for quantification method three

Method three produced a standard curve with efficiency of 110.3%, with a LOD of 3530 genomes and an error of 0.896. Therefore this method improved the sensitivity and efficiency of the assay, but the error value needed further improvement.

6.4.2.4 Bacterial quantification method four – qPCR of the *E. coli uidA* gene amplicon cloned into pGEM®-T plasmid vectors

As there was a large error value for the standard curve produced when genomic *E. coli* DNA was extracted and used in qPCR, despite acceptable efficiency, an alternative assay was used to improve performance using plasmid vectors which were beginning to become accepted as ‘gold standard’ for bacterial gene, and consequently bacterial quantification.

Method four involved insertion of a bacterial gene of interest in single copy into a plasmid vector, before using this ligated plasmid to transform cells. These cells were then incubated, with subsequent plasmid-amplicon DNA extraction and quantification of plasmid copies.

5µl of genomic DNA, extracted using the Promega Wizard® Genomic DNA Purification Kit (A1125), was used as template to generate a high concentration of *uidA* amplicons (size 61 bp and 186 bp) using *uidA* primer pairs F2 R2 and F1 R1 respectively (see chapter 5 table 5.1) with standard conditions (see sections 5.7.3 and 5.7.1 – 5.7.2).

6.4.2.4.1 Cloning of PCR products using pGEM®-T Promega vector systems

PCR products were ligated into pGEM® -T vectors using the Promega ligation kit (A3600, see section 5.8.1), followed by transformation of chemocompetent or electrocompetent cells as described in sections 5.8.2.1 – 5.8.2.3. Confirmation of successful transformation was by blue-white screen (5.8.2.4), qPCR, and restriction enzyme digestion (5.8.1 and 5.8.1.1 and figure 6.14), as a definitive way of demonstrating that the *uidA* amplicon was present in the plasmid. Successfully transformed bacterial cells were frozen down as clones for use as a long term source of plasmid DNA for standard curves. Plasmid DNA was extracted (5.8.3) and its concentration was measured, in order to perform the calculation to quantify *uidA* (and also 16S rRNA) amplicon-plasmid copy number.

6.4.2.4.2 Electrophoretic confirmation of *uidA* amplicon insertion in plasmid DNA

Following plasmid DNA extraction from cells, repeat qPCR was performed and the size of the amplicon was confirmed on an agarose gel (see sections 5.10). The results for *uidA* amplicons generated using *uidA* primers F2 R2 and *uidA* primers F1 R1 are shown in figure 6.13. This demonstrates that *uidA* amplicons of expected size were inserted into transformed cells

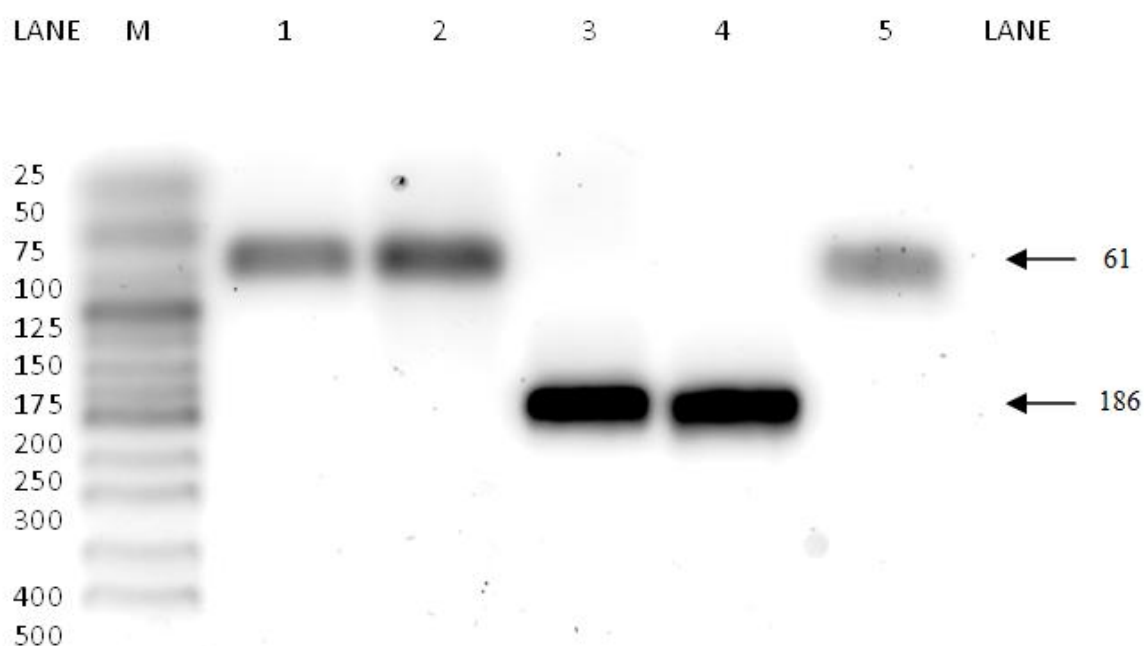


Figure 6.13 DNA electrophoresis following PCR amplification of plasmid DNA to confirm *uidA* amplicon insertion. 3.5% DNA agarose gel in TAE. Lane 1,2, and 5: Plasmid -amplicon 2 DNA. Lanes 3 and 4: Plasmid – amplicon 1 DNA. M: Marker lane, Hyperladder V, 25-500bp. Primers used to generate and confirm amplicon insertion were *uidA* primer pair F2 R2, with amplicon size 61 bp, and *uidA* primer pair F1 R1, with amplicon size 186 bp. See chapter 5 Table 5.1.

6.4.2.4.3 Restriction enzyme digestion to confirm *uidA* amplicon insertion in plasmid DNA

The method for restriction digestion is described in section 5.8.1.1, and this was followed by electrophoresis. The result is shown in figure 6.14. This technique was used with the plasmid into which *uidA* amplicon 2 DNA was cloned (figure 6.13). The

restriction enzymes cut on either side of the *uidA* amplicon within the circular pGEM-T plasmid. A single cut is produced by both enzymes, so that when a single enzyme is used a linear piece of DNA (3061 bp size) is produced, with two enzymes producing two fragments of DNA (3000, and 61 bp approximately, the sizes of the plasmid vector and the inserted amplicon). Control lanes include 'no restriction enzymes' and each enzyme used in isolation.

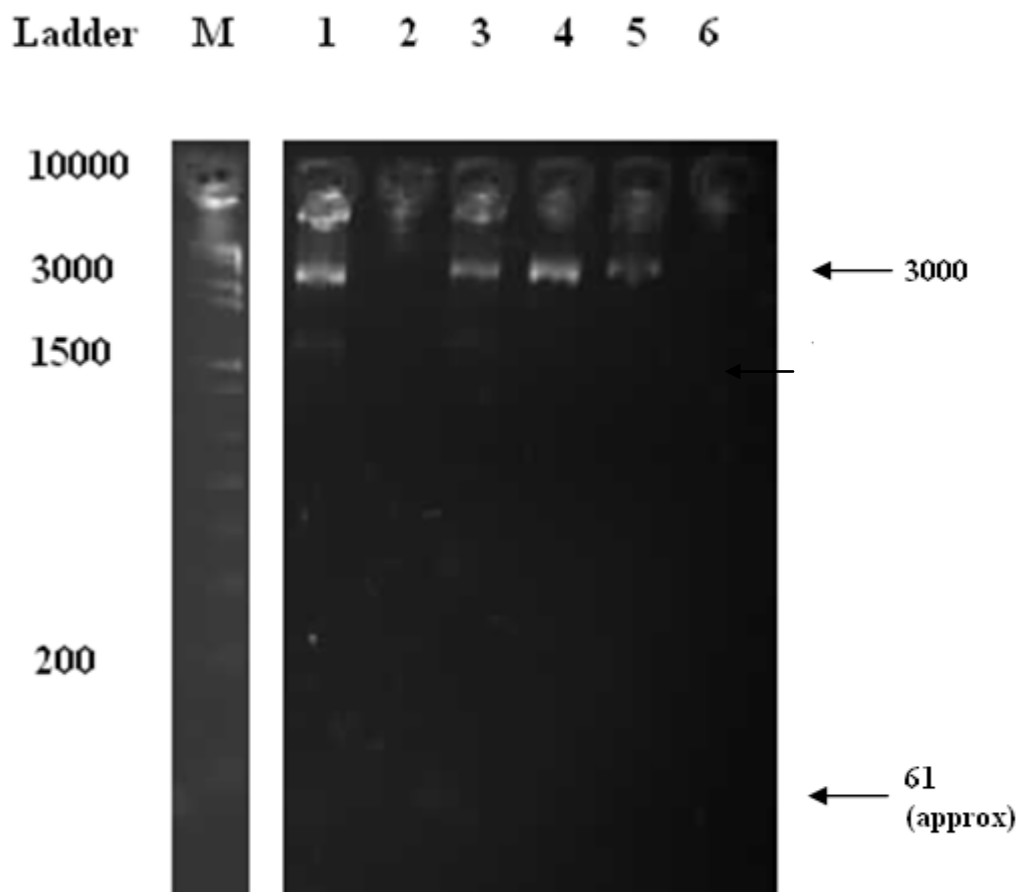


Figure 6.14 Restriction enzyme digest of plasmid *uidA* amplicon 2 DNA extracted from transformed electrocompetent cells. 2% DNA agarose gel in TBE, showing plasmid DNA digested using restriction enzymes (*SpeI* and *SacII*). Lane 1 and 3: Double restriction enzyme digest using both *SpeI* and *SacII* enzymes. Lanes 2 and 6: Plasmid DNA only, no restriction enzymes. Lane 4: *SpeI* enzyme alone, Lane 5: *SacII* enzyme alone. M: Marker lane, Hyperladder I, 200 - 10000bp.

6.4.2.4.4 *uidA* amplicon-plasmid copy quantification (examples)

Using this method, it was important to precisely calculate the amplicon-plasmid copy numbers in each DNA sample, and this was achieved by firstly calculating the molar

mass of the plasmid with the additional amplicon inserted (whichever amplicon this was), based on the known molar mass of a base pair and known amplicon and plasmid size. Amplicon-plasmid copies were then calculated using the expressions relating DNA mass (quantified with Nanodrop™), moles, molar mass, and Avogadro's number below.

A) Calculation based on the use of *uidA* primers F2 R2 to generate an amplicon.

The quantified DNA concentration for the *uidA* amplicon-plasmid was 122 ng/μl. The pGEM®T plasmid is 3000 DNA bp in size and when the *uidA* amplicon is inserted after ligation becomes 3061 bp in size.

- 1) Therefore RMM of the plasmid is 660 (M_r of a DNA bp) \times 3061 = 2.02×10^6
RMU
- 2) As moles = mass/RMM then $1.22 \times 10^{-7} / 2.02 \times 10^6 = 6.06 \times 10^{-14}$ moles per μl
- 3) Using Avogadro's no: molecules of plasmid-*uidA* insert = $6.022 \times 10^{23} \times 6.06 \times 10^{-14} = 3.65 \times 10^{10}$ copies plasmid-*uidA*/μl
- 4) Hence in 5μl of plasmid DNA there are $5 \times 3.65 \times 10^{10}$ copies plasmid = 1.83×10^{11} copies plasmid-*uidA* gene

All of the plasmid calculations are based on the reasonable assumption that the pGEM-T plasmid only accepts one amplicon into its ligation site.

(B) Calculation based on the use of *uidA* primers F1 R1 to generate an amplicon.

When the *uidA* primers F1 R1 were used to generate an amplicon, two plasmid DNA extractions were performed on the bacterial clones formed using the amplicon generated. The same principles as the above calculation were used with the important difference that the *uidA* amplicon-plasmid size was now 3186 bp.

6.4.2.4.5 qPCR of non linearised *uidA* plasmids

Plasmid DNA exists in multiple forms including supercoiled, nicked circular, and linear, which have different migration rates under electrophoresis, and can easily be purified separately from genomic DNA. Once the insertion of the *uidA* amplicons in plasmid DNA was confirmed, the extracted plasmid DNA was first evaluated in circular form. qPCR was performed as described in section 5.7.4.

When *uidA* primers F1 R1 were used to generate an amplicon (the *uidA* amplicon-plasmid size was now 3186 bp, 3000 bp plasmid + 186 bp amplicon) and transform cells, ultimately the plasmid DNA extraction produced a DNA concentration of 358.8 ng μl^{-1} . This resulted in *uidA* gene copy numbers of 5.14×10^{11} genes, when 5 μl of undiluted plasmid-amplicon DNA was used as template in qPCR.

The results for the fluorescence and standard curves produced by qPCR of non-linear plasmid DNA are shown in figures 6.15- 6.18. Results differed depending on the amplicon used. For amplicon 1, the assay gave a lower limit of discrimination from NTC samples of 5.14×10^8 genes, (figure 6.15) and the fluorescence signals from the probiotic bacterial controls were equivalent to those seen with NTC, or PCR

negative. The melt curve obtained from the qPCR of the plasmid *uidA* amplicon 1 DNA shows a unique product confirming the specificity of the reaction (figure 6.16).

When DNA containing the *uidA* amplicon 2 was quantified, the concentration for the *uidA* amplicon-plasmid was 122.37 ng/μl. The pGEM®T plasmid is 3000 bp in size and when the *uidA* amplicon is inserted after ligation becomes 3061 bp in size. Using the calculation described in section 6.4.2.4.4, 5μl of plasmid DNA has $5 \times 3.65 \times 10^{10}$ copies of plasmid = 1.83×10^{11} copies plasmid-*uidA* gene. When used in qPCR (figure 6.17) the lower limit at which non linear plasmid DNA template can be distinguished from NTC is 1.83×10^4 gene copies, or a DNA quantity of 61 fg μl⁻¹. The standard curve for this series is shown in figure 6.18.

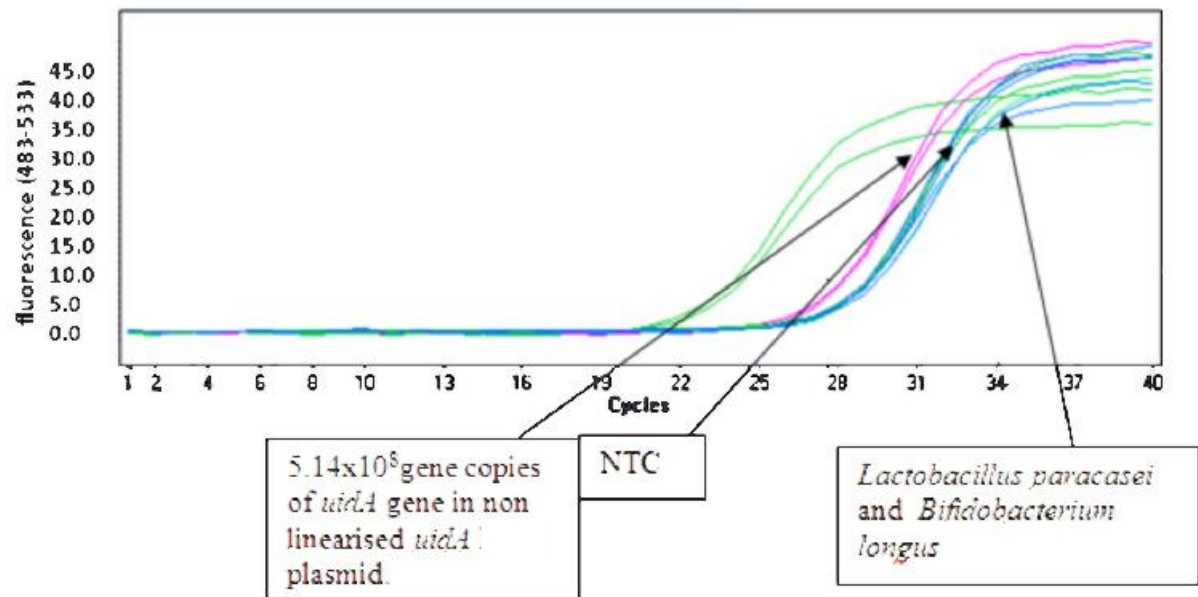


Figure 6.15 Comparison of the fluorescence curve generated by non-linearised plasmid *uidA* amplicon 1, NTC, and probiotic bacteria.

The pink fluorescent curve shows the lowest concentration of non-linearised plasmid *uidA* amplicon 1 template that could be visibly discriminated from NTC, i.e. 5.14 x10⁸ gene copies, or 1.79 ng DNA in 5µl. The fluorescence signals from the probiotic bacterial controls are equivalent to those seen in NTC, or PCR negative.

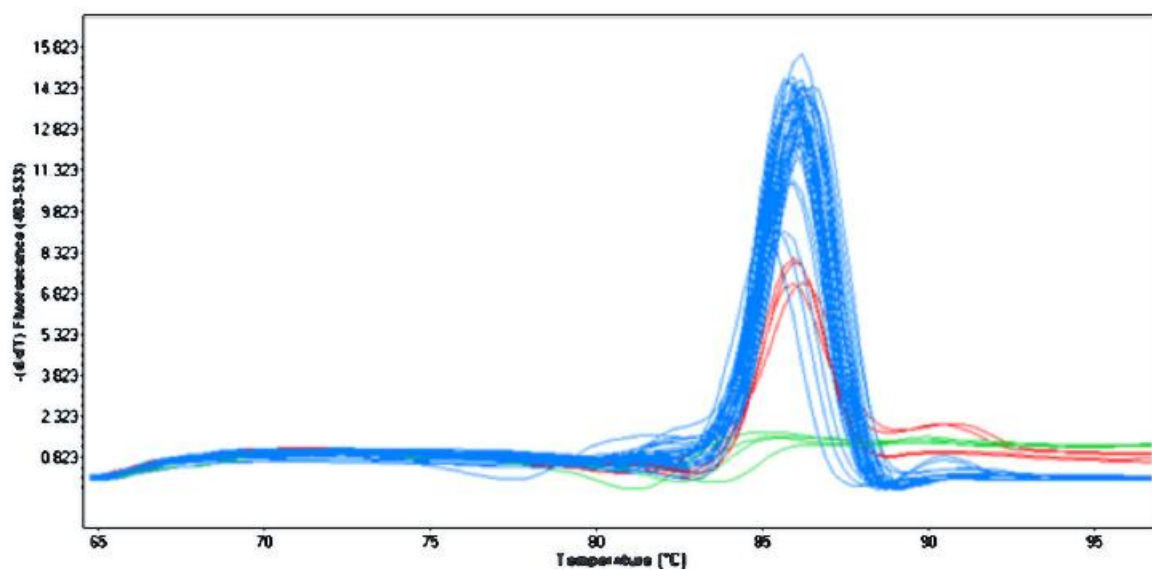


Figure 6.16 Melt curve obtained using *uidA* amplicon 1 plasmid DNA. Melt curve analysis of the *uidA* amplicon 1 plasmids shows the specificity of the qPCR product (i.e. a single peak).

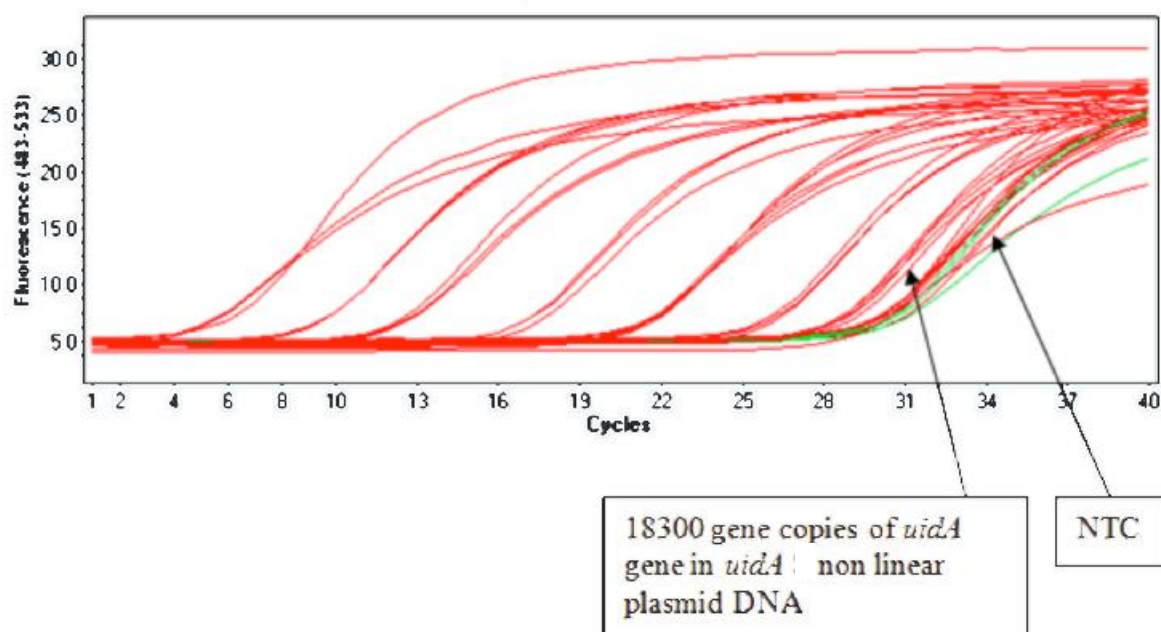


Figure 6.17 Fluorescence curves generated using plasmid *uidA* amplicon 2 DNA in qPCR. When plasmid *uidA* amplicon 2 DNA was assessed with *uidA* primers F2 R2, a lower LOD of 1.83×10^4 gene copies (or bacteria), or DNA concentration of $61 \text{ fg } \mu\text{l}^{-1}$ was seen.

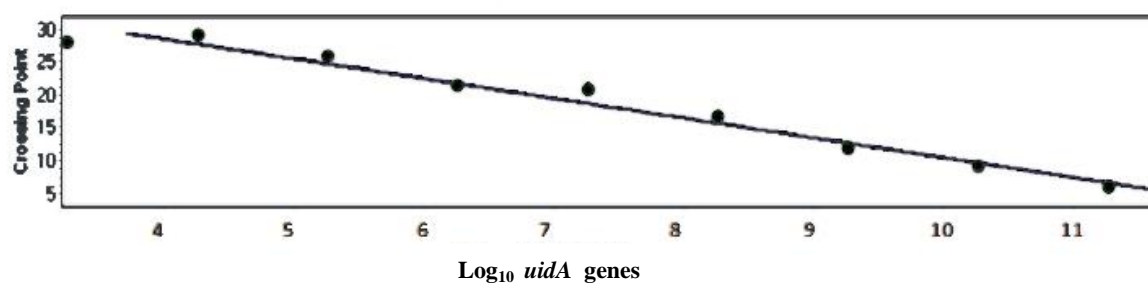


Figure 6.18 The standard curve generated from non linear plasmid *uidA* amplicon 2 DNA. Error 0.448, efficiency 2.158 (108%).

6.4.2.4.6 Conclusion for the use of method four with non linearised plasmid DNA

For plasmid DNA containing amplicon 1, the efficiency of the reaction could not be calculated for these templates, and the LOD was unacceptable for the assay at 5.14×10^8 gene copies. For plasmid DNA containing amplicon 2 the qPCR produced an error of 0.448, an LOD 1.83×10^4 gene copies, and an efficiency of 2.158 (108%), so despite improvements in efficiency and LOD, both the error and sensitivity needed further optimisation.

6.4.3 Linearisation of *uidA* amplicon containing plasmids. The definitive assay for *E. coli* quantification

The sensitivity of the assay was improved to the required level by linearization of the DNA template. When supercoiled plasmid DNA is linearized, the multiple plasmid DNA forms become identical in their linear structure. DNA physical template accessibility is greater and theoretically similar, and efficiency is improved compared to amplification from supercoiled plasmid forms (320), which may also therefore theoretically improve sensitivity. Figure 6.19 illustrates a representative example of linearization of plasmid DNA, in this case two separate samples of the same plasmid *uidA* amplicon 1 construct, one of which is successfully linearised.

The *uidA* amplicon 1 and *uidA* amplicon 2 plasmids were linearised with restriction enzymes (see section 5.9). Non-linearised plasmid DNA was electrophoresed alongside the linearised plasmid DNA in the gel electrophoresis as a control. When DNA ladders were used, their sizes were only applicable to linearised DNA, as the multiple forms of circular plasmid DNA migrate through agarose gel at

different rates which are not identical to their linearised sizes. Linearisation was a logical step as linearisation of plasmid DNA potentially increases template availability to qPCR reagents. Importantly, when plasmid DNA was linearised, the *uidA* gene quantity was diluted to one quarter of the pre-linearised amount (explained in section 5.9).

The linearised amplicon 1 plasmid was used for qPCR, and the same plasmid DNA was also assessed with the nested *uidA* primers F3 R3 (see table 5.1, chapter 5, and methods section 5.7.3. Similarly, the linearised *uidA*-amplicon 2 plasmid DNA was used in qPCR using *uidA* primers F2 R2 (section 5.7.1 and 5.7.2), and the results are shown in figure 6.20. The results were promising, so the qPCR was repeated to assess reproducibility of the *uidA* quantification assay and to allow assessment of coefficient of variation.

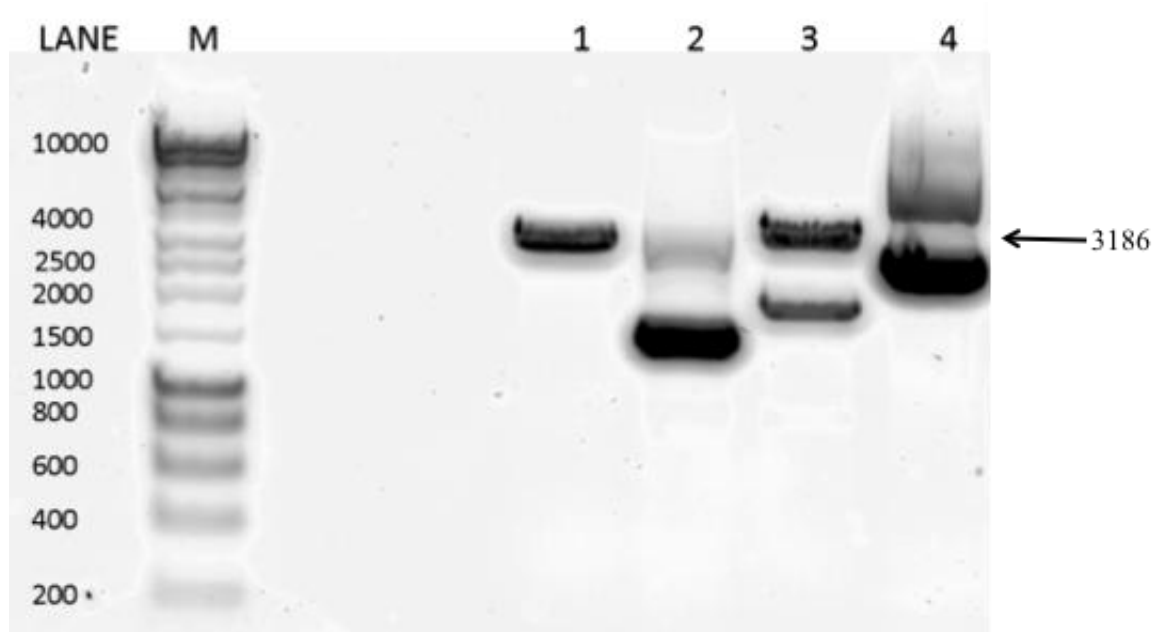


Figure 6.19 Figure showing representative examples of linearised *uidA* amplicon containing plasmid DNA. 1% DNA agarose gel in TAE.

Lane 1: Successfully linearised plasmid-amplicon 1 DNA. Lanes 2-4: appearance of non-linearised plasmid-amplicon 1 DNA. M: Marker lane, Hyperladder I, 200-10000bp.

Successfully linearised plasmid DNA is demonstrated as a single band, with a single restriction enzyme cut of plasmid DNA at 3186 bp as expected. Non-linear plasmid DNA exists in multiple forms and also cannot be directly 'sized' according to the hyperladder marker bands, which are linear. Single rather than the double band appearance is seen after a successful linearisation.

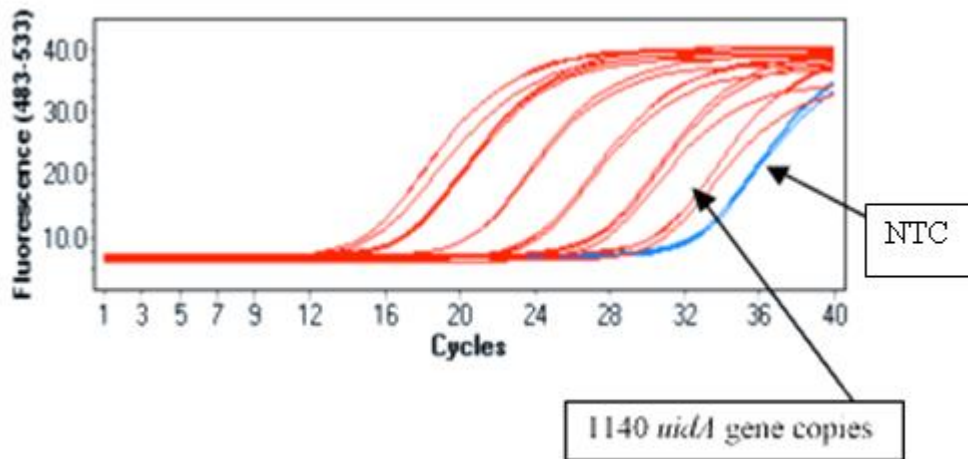
qPCR of the successfully linearised plasmid *uidA* amplicon 1 DNA produced increased sensitivity with a lower LOD of 2.11×10^5 *uidA* gene copies. The PCR efficiency value was extremely unacceptable though, at 29.87 (1494%) with an error of 0.841 (results not shown). The same plasmid DNA was assessed with the nested

uidA primers F3 R3 in an attempt to increase efficiency using this smaller amplicon. This gave the same lower limit of detection with unacceptable efficiency (338.2) with an error of 0.668 (results not shown). This assay was therefore also not useable.

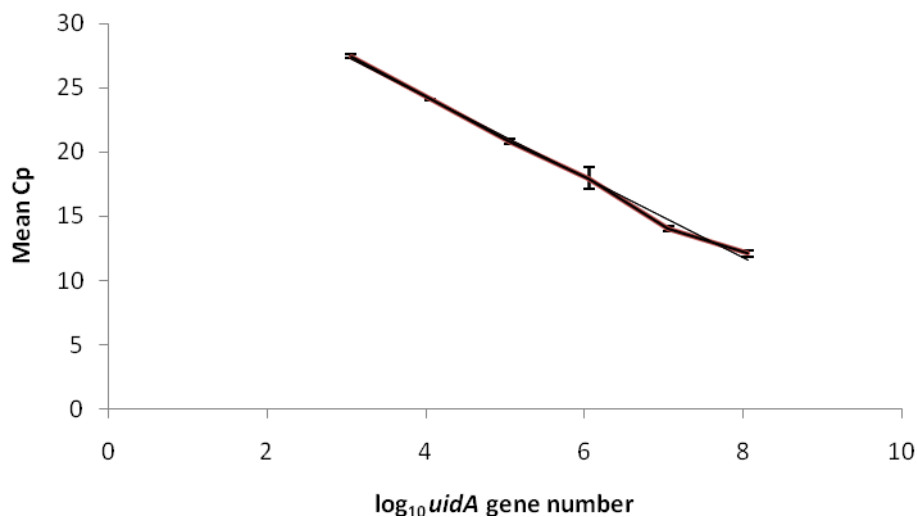
6.4.3.1 Use of the linearised plasmid *uidA* amplicon 2 DNA in qPCR to quantify *E. coli*, the definitive assay

The successfully linearised *uidA* amplicon 2 plasmid was used as template in the final standardisation experiment, to prepare suitable DNA standards by which *E. coli* bacteria could be accurately quantified from CD and control patient ileocolonic biopsies, in clinical trials (see chapter 7). This was a key experiment as it established an effective assay. Figure 6.20A shows the results obtained when the linearised plasmid *uidA* amplicon 2 DNA templates were used, and figure 6.20B shows the correlation coefficient between mean Cp and log₁₀ *uidA* gene number. Figure 6.20A demonstrates that 1140 *uidA* gene copies, and by inference 1140 *E. coli* bacteria, or a DNA quantity of 1.52 pg per 5 µl template, could easily be distinguished from NTC using these qPCR fluorescence signals. PCR efficiency (104%), error (0.130), and correlation coefficient (0.995) were all within acceptable limits.

On a second occasion the experiment was performed with the same templates the PCR efficiency was 2.132 (106%), error 0.096, and correlation coefficient 0.997, again within acceptable limits (not shown). Overall co-efficient of variability for Cp and log *uidA* gene numbers for the standard curve assay performed twice, n=6, was 3.72%.



A



B

Figure 6.20 Linearised plasmid *uidA* amplicon 2 fluorescence curves (A), and correlation co-efficient (B). The linearised plasmid *uidA* amplicon 2 DNA LOD was 1140 *uidA* genes with PCR efficiency of 2.07 (103.7%), error of 0.130 and correlation co-efficient (R^2) of 0.995. The 5 μ l plasmid DNA *uidA* templates shown represent 1.52 pg to 15 ng of DNA, or 1.14×10^3 to 1.14×10^8 gene copies. The blue line represents NTC PCR water. 1140 *uidA* gene copies = 1140 *E. coli* bacteria present, assuming 1 copy of *uidA* per genome in bacteria and one copy per plasmid DNA molecule.

A schema for the overall process used as ‘gold standard’ to absolutely quantify *uidA* and/or other future candidate genes is shown below

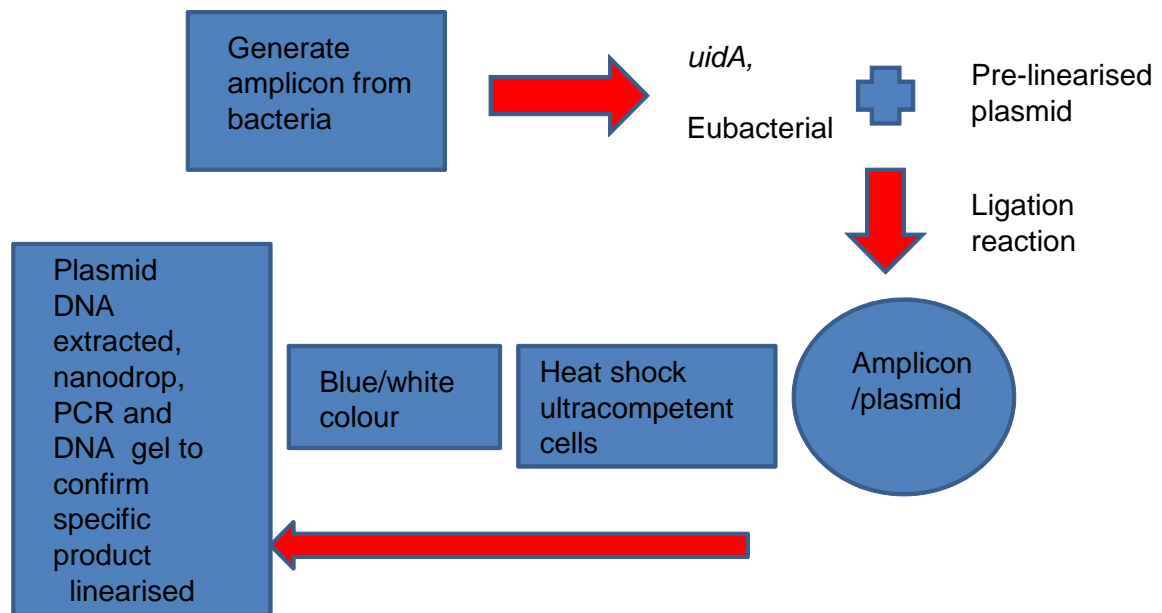


Figure 6.21 Schema of method for absolute quantification of bacterial gene numbers using plasmid-amplicon DNA to generate standard curves

6.4.3.2 Conclusion for the use of quantification method four with linearised plasmid DNA

qPCR of the linearised plasmid *uidA* amplicon 1 DNA produced a lower LOD of 2.11×10^5 *uidA* gene copies but with a PCR efficiency value of 29.87 (1494%) with an error of 0.841. The same plasmid assessed with the *uidA* primers F3 R3 gave the same LOD with unacceptable efficiency (338.2) with an error of 0.668.

Finally, qPCR of the linearised *uidA* amplicon 2 DNA demonstrated an LOD of 1140 *uidA* gene copies, PCR efficiency 104%, error 0.130, and a correlation coefficient of 0.995. with reproducibility (CV) <20%. When the standards were used to quantify bacteria in human biopsies, (see chapter 7), the same reproducibility in standard templates was seen between runs, so the main aim of the assay development described in this chapter was achieved. Thus the assay has been shown to have a sensitivity of 1140 *uidA* genes, or the lowest quantity of *E.coli* bacteria detectable in a human biopsy using this assay is 1140.

6.4.4 Application of method four to quantify total bacteria (16S rRNA genes)

As quantification method four was the most successful method for quantification of bacteria, this method was applied to quantify total bacterial numbers as a denominator for *E. coli* using the 16S rRNA gene primers and the pGEM®-T plasmid technology.

The same techniques as were used to generate the *E. coli* DNA standard curves were used to generate standard curves intended to be used to quantify eubacterial (total gastro-intestinal bacterial) numbers. A region of the 16S rRNA gene was used as a target for quantification as it is common to all bacteria. The 16S rRNA F/R primers (table 1, chapter 5) were used with the reaction conditions described in section 5.7.5 to produce the amplicon of interest which was ligated into the pGEM®-T plasmid. Any gastro-intestinal bacteria could be used to generate this curve, (as all have the

16S rRNA gene sequence used), but for convenience *E.coli* genomic DNA was used (as 5.3.3).

The ligated plasmid-amplicon DNA was used to transform ultra-competent cells (as per section 5.8.2.1) before plasmid DNA was extracted from these bacterial clones (as 5.8.3) and quantified. The 16S rRNA-plasmid DNA was then serially ten fold diluted for use in a standard curve. Once the 16S rRNA amplicon was generated from the plasmid DNA by qPCR, its size was confirmed by gel electrophoresis (section 5.10). The 16S rRNA gene copy number was calculated as per *uidA* using the equation in 6.4.2.4.4.

6.4.4.1 The effect of using different template volumes in the 16S rRNA qPCR quantification assay

Two different template volumes were used for this assay. The same template DNA dilutions were used, but in one assay 3µl volume template was used, and in the second 5µl. This was an attempt to see if DNA could be preserved. This could eventually be valuable when DNA was extracted from human biopsy samples. When 3µl of template was used, 2µl of PCR water was added as a volume control. It was expected that a high abundance of 16S rRNA genes would be present in colonic biopsies making this a feasible assay to attempt with lower template volume. In addition, the plasmid was also linearised, (see method section 5.9) and results (figure 6.22), to improve sensitivity.

6.4.4.2 Results for the quantification of total eubacteria (16S rRNA genes)

The 16S rRNA amplicon was successfully ligated into pGEM-T plasmid DNA and used to transform ultracompetent cells. The quantity of plasmid DNA extracted from these transformed cells at the initial attempt was 17.4 ng/μl and with a plasmid-amplicon size of 3200 bp, 4.961×10^9 plasmid-16S rRNA gene copies were present per μl of plasmid DNA in neat template.

When the diluted 16S rRNA plasmid DNA was used in qPCR to generate standard curves, the PCR quantification parameters were unacceptable, with the best result (see figure 6.23 for fluorescence and melt curves, the standard curve is not shown) seen with a 5 μl non-linearised plasmid DNA template. 5μl templates represented 2.48×10^{10} 16S rRNA genes or a DNA concentration of 87 ng in the most concentrated dilution.

In this case linearisation did not improve the assay sensitivity and the best result had a sensitivity of 2.48×10^6 bacteria or DNA quantity of 8.7 pg per 5 μl template. The efficiency was 1.831 (91.6%), with an error of 0.57. The results for 3 μl 16S rRNA-plasmid DNA and linearisation templates used in qPCR are not shown.

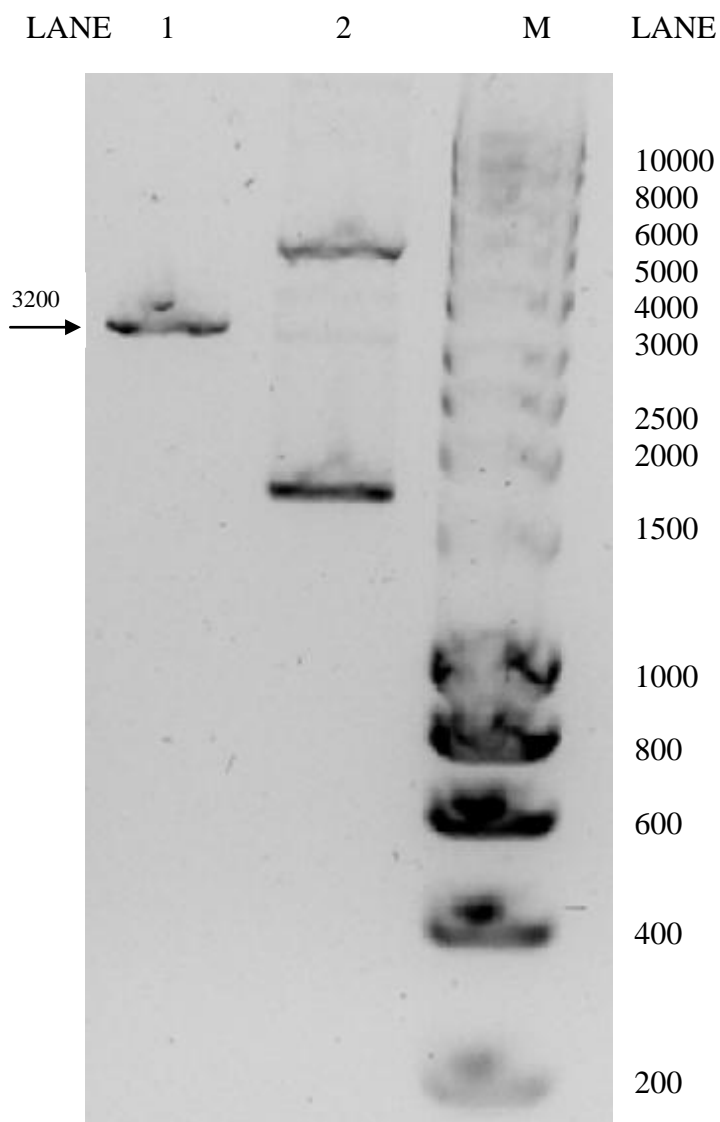
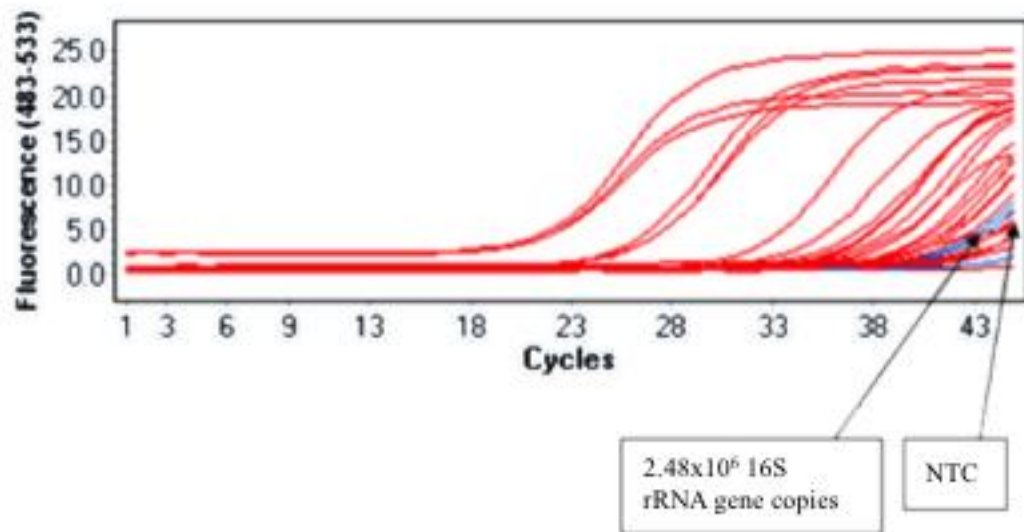
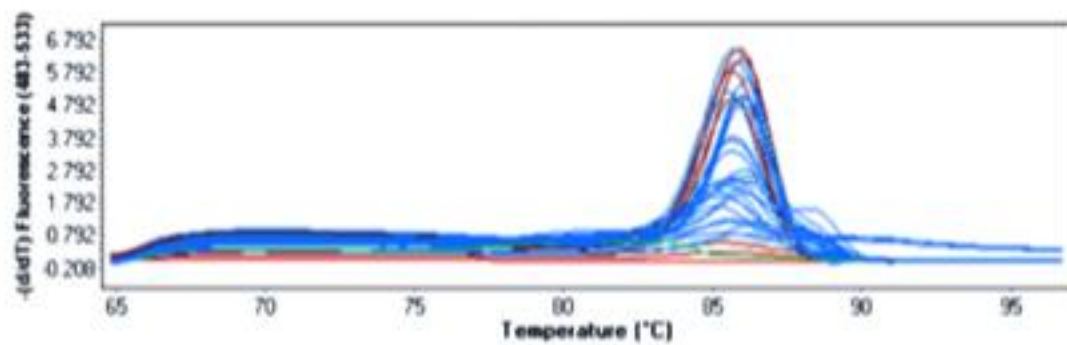


Figure 6.22 Linearisation of the 16S rRNA gene containing plasmid.

1% DNA agarose gel in TAE. Lane 1: successfully linearised 16S rRNA Plasmid-amplicon DNA. Lane 2: non-linearised 16S rRNA plasmid DNA. M: Marker lane, Hyperladder I 200-10000bp. Plasmid DNA containing the 16S rRNA amplicon exists in multiple forms (hence two bands) and does not equate to bp sizes in the linear hyperladder marker, but when linearised by restriction enzyme, exists as a single appropriately (3200 bp) sized product.



A



B

Figure 6.23 Best result for 16S rRNA gene eubacterial assay fluorescence curves

(A). The 5 μ l templates shown represent a range between 2.48×10^5 and 2.48×10^{10} 16S rRNA genes, or DNA quantities between 0.87 pg and 87ng. The lower LOD was 2.48×10^6 , with error of 0.57 and an efficiency of 1.831 (91.6%). The melt curve below (B), shows a unique product is formed when plasmid DNA is amplified by PCR.

6.4.4.3 Conclusion for the 16S rRNA total bacterial assay

The best result for the 16S rRNA assay had a sensitivity of 2.48×10^6 bacteria, which would probably have been utilisable for the expected total bacterial numbers in human biopsies (see section 6.2.1.1, an average magnitude of 4.84×10^7 bacteria would be expected per biopsy). The efficiency was acceptable at 1.831 (91.6%), but the error (defined in section 6.2.2.3) of 0.57 was not. This assay would require further optimisation before use with human biopsy tissue, for example optimising primer concentrations or amending Lightcycler® thermocycling conditions.

6.4.5 Method for the human cell denominator assay – quantification of 18S rRNA genes (human ribosomal genes) for biopsy standardisation

To standardise for biopsy size in the clinical trial, alongside mass measurement (after mucus removal), the 18S rRNA ‘housekeeping’ gene was used. It has been used previously for ileal biopsy standardisation (218), in order to express *E. coli* numbers per million 18S rRNA genes (or human cells). There is some question as to whether the 18S rRNA gene copy number would be increased in inflamed tissue independent of biopsy size as increased levels of inflammation are associated with increased numbers of non-epithelial human inflammatory cells, and for this reason standard biopsy wet weight was also measured. The 18S rRNA Eurogentec control kit (RT-CKFT-18S) was used as per kit instructions (see section 5.7.6).

6.4.5.1 Results for the human cell denominator (18S rRNA gene) assay

The commercial kit worked well, and was straightforward in comparison to the bacterial assays. The fluorescence curves generated by serial dilution of the human genome DNA on the first occasion are shown in figure 6.24, with the corresponding standard curve assessed by qPCR Cp curve fit points in figure 6.25A, and the correlation coefficient illustrated in 6.25B. A second run confirmed reproducibility, as did the templates' use in clinical trials (see chapter seven); the CV after the second run was 2.84%.

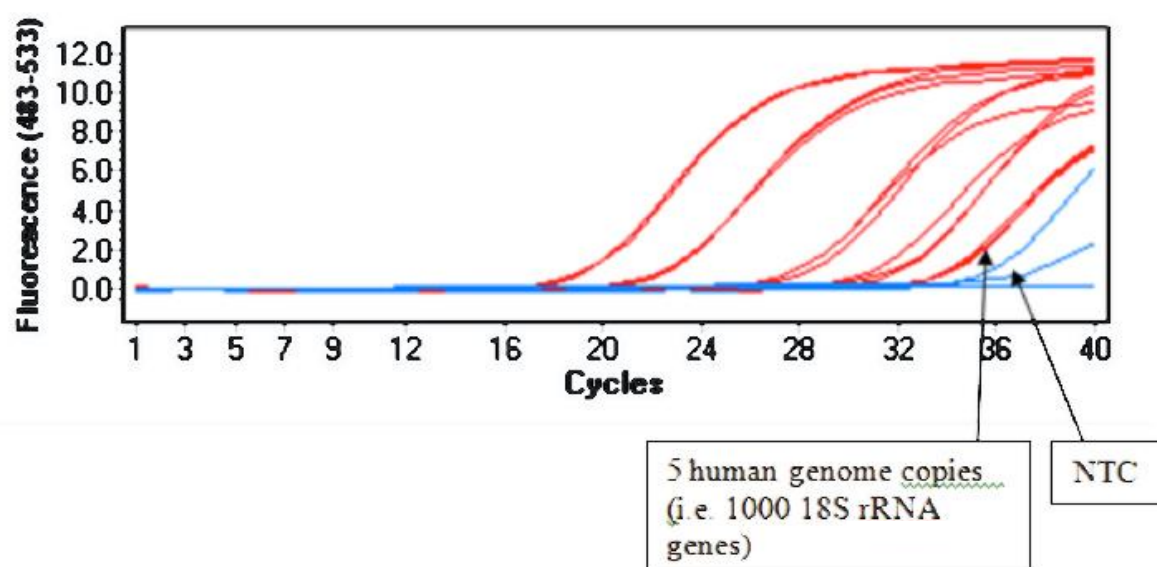
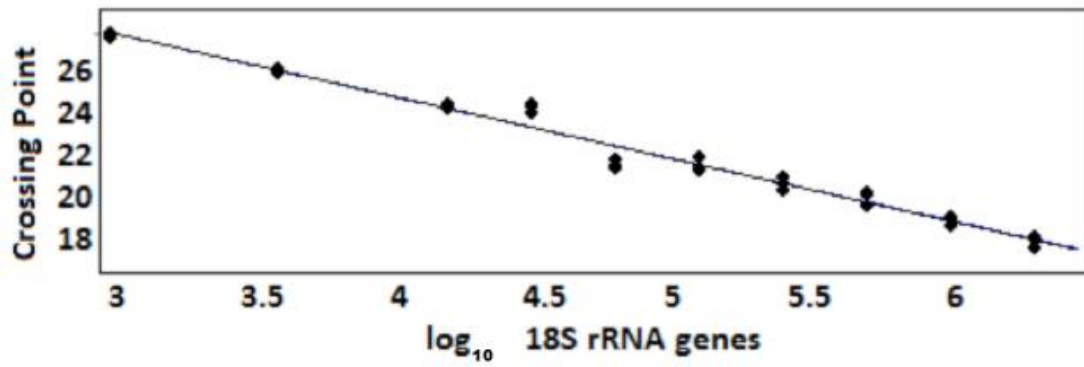
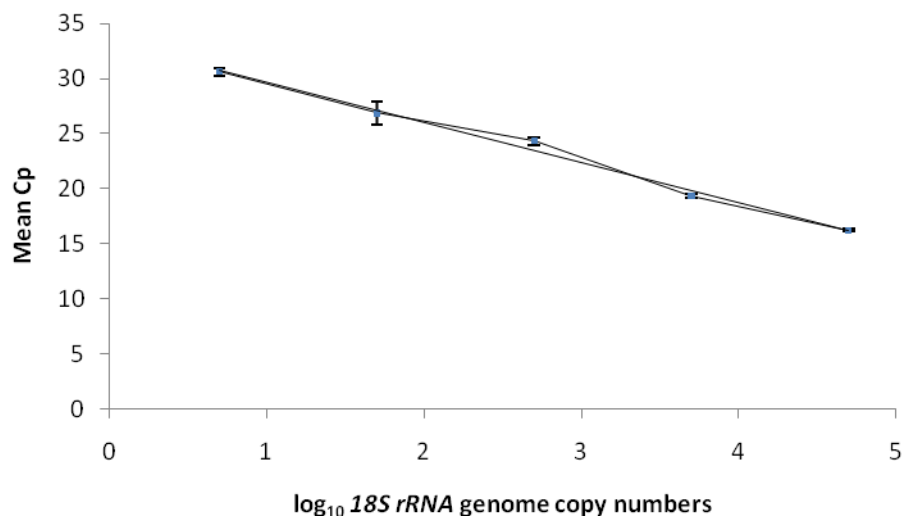


Figure 6.24 Fluorescence curves produced by the 18S rRNA genome standards.

Human genomic DNA (Increasingly dilute left to right, with template quantities of 5 to 50000 genomes or 1×10^3 to 1×10^7 18S rRNA gene copies shown). Blue curves show NTC (PCR grade water). The lower limit of detection is 5 genomes (200 copies of gene per genome)



A



B

Figure 6.25 standard curve derived from Cp's of fluorescence curves from 18S rRNA qPCR. Cp is plotted against \log_{10} of 18S rRNA gene number in these graphs.

(A) The standard curve produced has an efficiency of 2.074 (104%), and an error of 0.185. The calculated correlation coefficient is 0.992 (B)

6.4.5.2 Conclusion for the human 18S rRNA quantification assay

With a sensitivity of 5 human genome copies, efficiency 104%, error 0.185, correlation coefficient 0.992, coefficient of variability 2.84% and reproducibility <20%, the human 18S rRNA assay met all requirements for use in the clinical trials (see chapter 7).

6.5 Summary of qPCR assay performance

The statistical performance of all of the assays used in this chapter to quantify *E.coli* bacteria, whether based on CFU counting as a component, calculation of quantities using published genome sizes, or the use of modified plasmid techniques for quantification, are summarised in table 6.3. These statistical parameters are defined in section 6.2.2. The optimum statistical performance of the 16S rRNA and human 18S rRNA assays are summarised in table 6.4.

Summary Table 6.3 Performance of assays for *uidA* genes

The assays were initially assessed on the criteria of sensitivity, efficiency, and qPCR error, and where these were inadequate, the correlation coefficient, and CV (or reproducibility) of the assay were not assessed. Inter-assay CFU CV was assessed as it was informative at an early stage.

*** means that the assay was used for tissue biopsies in clinical trials.

Method	Sensitivity	Efficiency %	Error	Correlation coefficient	Coefficient of variability %
Crude extraction DNA, Method 1	73.3×10^5 CFU	59.1	0.383		18 for CFU plating
Qiagen kit DNA extraction, Method 2	72.2×10^5 CFU	141	0.298		18 for CFU plating
Use of bacterial genomes, Method 3	3530 genomes	110.35	0.896		
Method 4 – non linear plasmid <i>uidA</i> amplicon 1	5.14×10^8 genes				
Method 4 – non linear plasmid <i>uidA</i> amplicon 2	1.83×10^4 genes	108	0.45		
Method 4 -Linearised plasmid <i>uidA</i> amplicon 1	2.11×10^5 genes	1494	0.841		
Method 4 – Linearised plasmid <i>uidA</i> amplicon 1, nested primers	2.11×10^5 genes	16910	0.668		
Method 4 – Linearised plasmid <i>uidA</i> amplicon 2 ***	1140 genes	104	0.130	0.995	3.72

Summary Table 6.4 Performance from the 16S rRNA gene and 18S rRNA gene assays

The assays were initially assessed on the criteria of sensitivity, efficiency, and qPCR error, and where these were inadequate, the correlation coefficient, and reproducibility (CV), of the assay were not assessed.

*** means that the assay was used for tissue biopsies in clinical trials.

Method	Sensitivity	Efficiency %	Error	Correlation coefficient	Coefficient of variability %
16S rRNA genes (plasmid amplicon)	2.48x10 ⁶ genes	91.6	0.57		
18S rRNA genes control kit ***	5 genomes (1000 gene copies)	104	0.185	0.992	2.84

6.6 Discussion

This qPCR assay was developed to examine the quantities of *E. coli* in colonic tissue from areas of inflamed and uninfamed intestinal biopsy tissue from patients participating in clinical trials which would include periods of relapse and remission. This would also afford the opportunity to quantify change in *E. coli* numbers with histological findings and clinical state (see chapter 7).

The development of an assay for absolute quantification of *E. coli uidA* genes (and hence *E. coli* bacteria) using qPCR, involved a lengthy process of evaluation of multiple techniques. The crucial issues were assay sensitivity and accurate DNA and gene copy quantification.

6.6.1 Sensitivity and absolute quantification

In a previous study molecular quantification (218) of *uidA* genes in CD ileal and colonic tissue generated $0.5\text{--}5 \times 10^6$ *uidA* genes per million human cells in ileal biopsies and approximately 1.25×10^4 cells in colonic tissue, with 0.75×10^4 *uidA* genes in healthy tissue. The biopsies used in this work, which also involved mucus removal, unlike the previous study (218), overall produced an average of 187000 18S rRNA genes per biopsy, or 937 human genomes (cells) per biopsy (as there are 200 18S rRNA genes per human genome). Basing calculations on the results of (218) which they expressed as *uidA* genes /million human cells, one would expect 469–4690 *uidA* genes from DNA extracted from ileal tissue and 11.7 *uidA* genes per biopsy of colonic tissue, with 7 *uidA* genes in control tissue for average biopsy size. When human biopsy tissue had been analysed, standard biopsy mass (wet weight) was found to be

between 8-115 mg at the extremes of sampling in clinical trials evaluating CD and control tissue biopsies (n=54, mean mass 48.4 ± 3.8 mg SEM (Chapter 7).

In terms of quantifying bacteria using CFU, the numbers likely to be of interest in CD, identified from previous laboratory work were 0 – 3000 CFU per biopsy (232) but these figures include all aerobic bacterial species cultured and not simply the *E. coli* identified specifically by the *uidA* gene.

In one study looking at *uidA* genes in twins (226), the *uidA* gene numbers are expressed per 10^6 16S rRNA genes, at levels of log 1.3-4.5 (or 20-31600), but the authors state that this is when *E. coli* was detectable, and that levels of *E. coli* were significantly higher in patients with ileal CD than colonic CD. In support of this work however, the authors also conclude that when *E.coli* was detectable for either colonic or ileal disease it was present at all areas of the gastrointestinal tract (ileum, ascending colon, transverse colon, descending colon and rectum). This serves to illustrate that often different studies use a different denominator, which makes direct comparison difficult for microbial ecology and absolute quantification, and also suggests that the *uidA* genes were sometimes undetectable. Without knowing the average 16S rRNA gene numbers present in a biopsy in CD tissue, it was difficult to know what *E. coli* numbers to expect and plan assays for. In healthy control tissue in the same (226) study *uidA* genes were detected but only, where detected, at levels of log 1.2 -2.5 (or 15.8- 316) per 10^6 16S rRNA genes. Significant variance was clearly seen as in one twin pair with ileal CD, one individual did not have *uidA* genes identified at all. 16S rRNA gene copies were also expressed in the twin study per β -globin gene present. Whilst it is valid to use a housekeeping gene, the differing denominators for each gene quantity expressed make direct comparison difficult, and ultimately one of the

improvements of the assay in this work is that it could be directly compared with most studies as each component examined was independently absolutely quantified.

Utilising FISH gives at best semiquantitative answers. One study (311) produced FISH images for various categories of mucosal bacterial concentrations, but in the results the authors remarked that accurate quantification could not be achieved, particularly in the tissues with high bacterial concentration. In the same study 16S rRNA genes were looked for with 10^3 - 10^6 CFU/l found, but not in all patient groups and generally in less than 83% of patients. Analysis of the spatial arrangement of bacteria and a visual representation of their abundance is certainly possible (218), but the data in the Swidsinski study are not obtained using absolute quantification, and are categorical data. This data gives only a broad indication of potential bacteria present (<1000, 1000-10000, and >10000 CFU/ μ l). Other authors have attempted to quantify *E. coli* and other bacterial numbers by specifying the % of biopsies examined with the specified bacterial species present within the tissue, without quantifying the actual quantity within each biopsy (200). This is also therefore a categorical measure. Eventually it was decided that the best solution was to aim for the maximum sensitivity possible for an absolute quantification assay (three bacteria), but that acceptable figures would be those approximating 1000 bacteria with acceptable reproducibility or CV (aim <20%). In terms of PCR performance targets were efficiency between 90-110%, error <0.2 and correlation coefficient >0.95, which were all eventually achieved. This was very difficult and pushed the limit of detection for qPCR to the extreme.

The first quantification method resulted in a very poor standard curve. This may have been due to the crude method of DNA extraction used, which would have given even lower yield when each bacterial suspension was diluted. The fact that CFU

counting can be inherently variable did not help this attempt at the assay. The assay was clearly improved using method two when an efficient commercial kit for DNA extraction was used, but this still resulted in an unacceptable assay that was not sensitive enough, with poor efficiency and error, key factors in PCR.

With a switch to purely molecular techniques there was removal of some of the variability seen when using methods involving CFU counting. The published genome size of *E. coli*, along with accurate quantification of extracted DNA using the Nanodrop TM, allowed good approximation of bacterial quantities, with a technique similar to (306). Unfortunately, this ultimately produced an assay with unacceptable error despite good efficiency and lower limits of detection (3530 copies).

6.6.2 The successful development of a PCR method for bacterial quantification in human intestinal biopsy tissue

Finally, the use of plasmid technology produced the definitive solution. The multi-step process of producing a suitable amplicon, splicing it into DNA, transforming cells to accept plasmid DNA and ultimately extracting the plasmid DNA from these clones and confirming the presence of the amplicon by qPCR with or without restriction enzyme digest was complicated, but meant that bacterial numbers could be quantified with great precision using these standards for our template DNA. This was rewarded when an assay was produced with appropriate sensitivity, efficiency, reproducibility, and correlation coefficient. The linearisation of the plasmid template proved important for the *uidA* assay, but irrelevant to the attempted 16S rRNA gene assay. This technique is regarded as the gold standard currently (309;310) and it is in general vital to linearise plasmid DNA, as plasmid secondary

and tertiary structure can preclude against sensitive target sequence annealing. The ‘gold standard’ technique of inserting a gene of interest into a plasmid and using this to construct a standard curve for target sequence copy number can also produce an over-estimation of gene numbers if the plasmid DNA is left in the supercoiled circular non-linear form, because PCR amplification is less efficient when DNA is in this form (320) in the early stages of PCR amplification.

6.6.3 Definition of the lower limit of detection

It became clear that it was important to define a lower limit of detection for the assay and this was simply defined as the limit where a fluorescence curve for a given template could be clearly and repeatedly differentiated from a PCR non-template water control and also form part of a set of repeatedly useable templates that satisfied the statistical requirements for the PCR assay. The difficulties involved in looking at the lower limit of detection in qPCR are discussed in the literature. The use of low copy number template (317) problems with NTC, its non normal distribution and the artificial assignment of an end cycle number are subjects of current review (321). qPCR data is ‘non-normal’, censored and heteroscedatic (314) and its limit for magnitude of low copy detection is 1000 fold lower than standard endpoint PCR which argues against running gel electrophoresis to delineate LOD after qPCR has been performed. All appropriate statistics and methods are provided so that these assays can be reproduced, consistent with good qPCR practice and MIQE standards.

6.6.4 Utilisation of plasmid technology for 16S rRNA gene quantification

Given its successful application to *uidA* quantification, it was appropriate to use the plasmid technology for other assays. The 16S rRNA gene assay reached the stage of development where a suitable clone had been produced for plasmid DNA and linearised, but when the plasmid DNA templates were used the qPCR did not reach acceptable performance statistics. Primer selection (as seen when different concentrations of primers were used with the *uidA* assays) and alteration of qPCR reaction conditions could improve this, though template quantity did not prove a crucial factor. Optimisation of this assay would provide useful denominator quantification of all bacteria present in biopsy DNA, and allow assessment of bacterial subset percentage changes in CD human tissue at different longitudinal timepoints and at different levels of disease activity.

6.6.5 The use of the 18S rRNA assay for standardisation of human cell numbers

In contrast to the other assays the human 18S ribosomal RNA assay, which had been used in a previous study (218), was straightforward. Its use as a housekeeping gene to standardise human tissue amounts was combined with standard biopsy mass measurement when CD biopsies were processed. The inflammatory cells involved in active CD lesions may produce an increase in 18S rRNA gene numbers which may be disproportionate to actual biopsy mass, so it was important to make both these measurements to allow for assessment of any disparity.

6.7 Conclusion

After a long, difficult process of development, the use of linearised plasmid vectors to quantify *E. coli* bacteria proved the most sensitive, accurate and reproducible method used. Selection of appropriate primers to amplify a segment of the *uidA* gene, and accurate DNA quantification, also proved key. Molecular based techniques that did not include CFU enumeration proved more useful than those which did. Both the *E. coli*, and human 18S rRNA gene assay used as a denominator, fulfilled the statistical requirements specified for these assays, and were therefore used to perform absolute quantification on CD and control intestinal biopsy tissue in the plantain clinical trial and aphthous ulcer study (chapter 7).

CHAPTER SEVEN : QUANTIFICATION OF MUCOSA-ASSOCIATED BACTERIA IN CROHN'S DISEASE DURING RELAPSE AND REMISSION

7.1 Hypotheses:

As aphthoid ulcers are probably the earliest lesions seen in active CD, the putative causative agent of CD, adherent, invasive *E. coli*, will be increased in number in these lesions in both human ileal and colonic tissue compared with control tissue.

As any invading bacteria, including *E. coli*, are likely to invade through the specialised M cells in the epithelium overlying Peyer's patches and lymphoid follicles, the aphthoid ulcers containing bacteria are most likely to be found overlying Peyer's patches or lymphoid follicles.

Greater quantities of *E. coli* can be expected to be present in tissue where patients show increased disease activity and when they have macroscopic or microscopic intestinal inflammation.

Ingestion of soluble plantain fibre, by blocking recruitment of bacteria to the gut epithelium may be associated with a reduction in mucosa-associated *E. coli*.

7.2 Aim:

To utilise qPCR to evaluate the number and distribution of bacteria within CD and control tissue, at different stages of disease activity.

7.3 Section A. Effect of dietary Soluble Plantain fibre on mucosal bacteria in Crohn's disease

The hypothesis that bacterial-epithelial interactions may be lectin-carbohydrate mediated, and may be inhibitable by soluble plant fibres is currently under ongoing investigation by our group. A number of soluble plant fibres, including particularly soluble plantain (banana) fibre, have been shown to inhibit adhesion of *E. coli* to the intestinal mucosa (232) as well as the translocation of CD *E. coli* across M cells and follicle-associated epithelium, the likely portal of entry for AIEC in CD intestinal tissue (322). This has obvious potential therapeutic benefit in arresting the events that induce mucosal inflammation in CD. In areas of the world where plantain fibre is consumed in large amounts, such as Africa, India and the West Indies, CD is rare. These in-vitro and epidemiological observations have led to the development of a randomised double blind placebo controlled clinical trial of plantain fibre supplementation to CD patients at this institution (University of Liverpool and the RLBUHT NHS Trust). This allowed investigation by analysis of clinical trial biopsies using the qPCR methods developed in chapter 6, correlated with endoscopic, histological, and clinical assessment.

7.3.2 Methods

7.3.2.1 Randomised double blind controlled trial of soluble plantain fibre in maintenance of CD remission

This trial had full ethics approval (EudraCT number: 2006-005526-23, REC reference : 07/H1002/105, ISRCTN number 62819212. Ethics committee Northwest 2 (Liverpool Central) Protocol code number NSP/0002/07 and RLBUHT NHS Trust approval).

Patients with CD were recruited based on the inclusion and exclusion criteria in 7.3.2.2. They were randomised independently to receive either 5g plantain fibre, or maltodextrin placebo twice daily over twelve months, with an option to be included in the biopsy arm of the study which involved sigmoid biopsy collection at 0 and 3 months, or at a point earlier than 3 months if a relapse or decision to withdraw from treatment occurred (withdrawals and relapses are documented in appendix five). Patients were also stratified into groups receiving immunomodulators (azathioprine, mercaptopurine or methotrexate) and those receiving none, and by location of CD; i.e patients with colonic disease and those with ileal tissue involvement. Three to four additional biopsies were collected from the sigmoid alongside those taken for standard histology, and were analysed by the qPCR techniques developed in chapter six to quantify *E.coli* numbers standardised against human cell numbers present in the biopsies. The patients recruited to the trial had assessments of disease activity, assessed with the Crohn's Disease Activity Index (323) or CDAI at 0,1,2,3,6, 9 and 12 months, or if there were any symptomatic concerns. Relapse was defined as CDAI

> 220, and remission as CDAI < 150. Macroscopic sigmoid appearance and standard histology were assessed alongside CDAI and qPCR quantification. 12 patients in total were recruited to the biopsy arm of the clinical trial of which 2 withdrew, and one relapsed; one of the withdrawal patients was clinically relapsing also. qPCR assessment of large intestinal biopsies was performed blind of any clinical information, see results section 7.3.3.3 – 7.3.3.5

7.3.2.2 Inclusion and exclusion criteria for the plantain trial

The inclusion criteria were as follows:

- 1) Patients with CD as diagnosed by conventional clinical, radiological and histological criteria.
- 2) CD involving small bowel, colon or both.
- 3) CD that is in remission: Crohn's Disease Activity Index (CDAI)
< 150
- 4) Patients who have had a relapse of disease within the previous 12 months (Harvey Bradshaw Index modified for retrospective use >4, and/or a symptomatic relapse requiring a change in CD treatment.

The exclusion criteria were as follows:

- 1) Patients under 18 years or unable to give informed consent.
- 2) Patients who have had surgery for CD within the previous 12 months.
- 3) Any change to medication for CD within the previous 3 months.

- 4) Patients receiving corticosteroids or infliximab within the previous 3 months.
- 5) CDAI >150
- 6) Patients currently receiving enteral nutrition as part of their treatment for Crohn's disease.
- 7) Participation in other trials in the last 3 months.
- 8) Women intending to become pregnant in the next 12 months
- 9) Patients with a known allergy to bananas

7.3.2.3 Biopsy sampling, macroscopic assessment of tissue, laboratory processing and analysis of qPCR data in the plantain trial

Biopsies were taken from the sigmoid colon up to 15-25 cm (rectosigmoid) from the anus, and patients did not receive bowel preparation for this. These samples were in addition to any that were clinically indicated.

The macroscopic appearance at endoscopy was documented (see table 7.1), for later comparison with microscopic appearance and *uidA* gene quantification from biopsies at the same region of the gastro-intestinal tract. A standard Olympus endoscope was used and biopsies were taken with standard disposable endoscopic forceps.

7.3.2.4 Routine histological assessment of tissues

The microscopic appearance of biopsies taken as part of routine clinical care were routinely examined by pathologists from the pathology department at the RLBUHT

hospital following standard formalin fixation, embedding and sectioning with routine haematoxylin and eosin (H and E) staining, and reported (see table 7.1).

7.3.2.5 Laboratory processing of biopsies

Samples were processed as described in Methods section 5.11, and DNA was extracted using the techniques described in 5.3.2. Once DNA was extracted from colonic tissue samples and their mucus washings, the number of *uidA* gene copies, and therefore *E. coli* bacteria, in each sample could be quantified using the qPCR assay developed in Chapter six. Aliquots of DNA (5 µl) were used as template in triplicate qPCR reactions to detect average *uidA* quantity as a marker of *E. coli* numbers in tissue. qPCR for human 18S rRNA was used (as described in section 5.7.6) to quantify human cell numbers, and *uidA* gene copies were expressed relative to one million 18S rRNA genome copies. Biopsy samples were weighed prior to qPCR so that *uidA* gene copies could also be expressed per mg of biopsy tissue (wet mass).

7.3.2.6 Reproducibility of the qPCR assays

In each qPCR assay, for every sample analysed, whether for *uidA* gene or 18S rRNA gene quantification, one of the standard templates from the *uidA* or 18S rRNA gene standard curves developed in Chapter six (sections 6.4.3.1 and 6.4.5) was included in triplicate in the run, with the same automated LightCycler® settings applied as were used in the original standardisation runs. The quantified standard template amount

was compared to its original value in the standard curves to assess reproducibility of measurements, and the range of variation is explained in the results (section 7.3.3.2).

qPCR quantification was initially performed separately for biopsy tissues and the mucus washings from these, and then quantities were combined. Analysis comparing remission and relapse biopsies was performed on all groups using Wilcoxon's two sided signed ranks test, with data considered significant for $p < 0.05$.

7.3.3 Results

7.3.3.1 Clinical, Endoscopic, Histological, and Physical data

There was a large amount of clinical, histological and quantitative data produced in the clinical trial of plantain therapy, which is summarised in table 7.1. Of note, three patients withdrew or had clinical relapse at the time of preparation of this thesis (see figure 7.1, code numbers 502, and 603, with patient 101 withdrawing at the point of near clinical relapse) had macroscopically normal endoscopies, with histology also unremarkable. Similarly, patient 604 had mild proctitis on endoscopy but normal histology and CDAI scores (table and figure 7.1). Conversely, there was a patient (201) who had histological features of inflammation within the sigmoid including crypt abscesses and granulomata in the lamina propria, but who had normal endoscopies on two occasions and indeed remained clinically well throughout the trial. Also patient 504, the only patient to test positive for *Clostridium Difficile* Toxin, or in fact any bacterial agent, remained clinically (see figure 7.1), macroscopically, and microscopically quiescent.

From the physical parameters measured, of note the extracted DNA quantities were always much lower in biopsy samples from patients who had relapsed (see table 7.1), which may reflect changes in human or bacterial DNA quantities in the sigmoid colon in CD relapse, though only small numbers were involved. DNA quantity decreased despite average wet biopsy mass increasing in relapse in patients 101 and 502 (not 603) which may support the DNA change being due to bacterial changes.

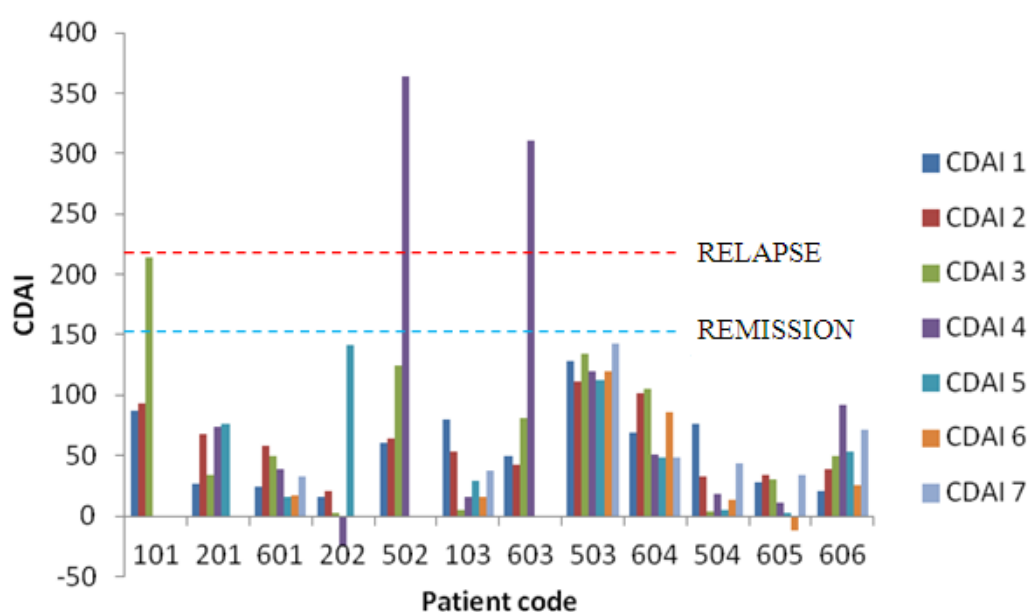


Figure 7.1 Disease activity levels for patients in the biopsy arm of the plantain clinical trial, assessed by CDAI. Changes in CDAI for the 12 patients in the biopsy arm of the plantain trial at different timepoints are shown (CDAI 1-7, corresponding to time from recruitment (month 0) until months 1,2,3,6,9 and/or relapse, withdrawal, or completion of the trial (twelve months of maintenance plantain therapy). The dashed red line indicates CDAI primary endpoint for relapse (>220), and the dashed blue line indicates CDAI for patients in clinical remission (CDAI <150). As the trial is ongoing all patient data are blinded for plantain/placebo.

Table 7.1 Physical characteristics and clinical data for patients in the biopsy arm of the plantain clinical trial. Details shown include patient code, average quantity of DNA extracted from biopsies (and mucosal washings in brackets), macroscopic appearance of the sigmoid at endoscopy at 0 and 3 months or relapse (in brackets), histological appearance at 0 and 3 months or relapse (in brackets), average biopsy mass from patient at 0 and 3 months or relapse (in brackets), and results of screening stool culture for any infectious organisms. REL = relapse, WDL = withdrawal. Patient 101 withdrew near the point of clinical relapse and 603 was clinically relapsing at the time of withdrawal. Colours: red = withdrawal/relapse, lavender = 3 month data.

Patient code	DNA ng/ μ l (average)	Macroscopic Appearance	Microscopic appearance	Ave Biopsy wet mass/mg	Stool Culture
101 (WDL)	84.7 (7.8) 48 (7.47)	Normal (Normal)	Normal(Normal)	27 (59)	Negative
201	74.6 (12.6) 198 (14.9)	Normal (Normal)	chronic inflammation/granulomata (active CD, cryptitis, crypt abscesses and epitheloid granulomata in the LP)	100.5	Negative
103	67 (7.75) 59.5 (4.82)	Normal (Mild proctitis)	Normal (normal)	26	Negative
502 (REL)	79.7(12.6) 24.8 (5.86)	Normal (Normal)	Normal (Normal)	23 (56.3)	Negative
601	109.1(9) 224 (5.83)	Normal (Normal)	Normal (Normal)	108	Negative
202	110.7 (4.59) 113 (6.92)	granularity (Normal)	Normal (Normal)	-	Negative
603 (WDL)	123 (4.55) 20.7 (7.75)	Normal (Normal)	Normal (Normal)	85.8 (37.3)	Negative
503	71.3 (8.2)103 (34.5)	Normal (Normal)	Mild increase in muciphages (Normal)	111 (15)	Negative
504	165(13.5) 108 (98.1)	Normal (Normal)	Normal (Normal)	-	C Difficile + (screen)
604	148(8.73) 124(18.4)	Mild proctitis (Normal)	Normal (Normal)	-	Negative
605	77.7(6.61) 147 (8.72)	Normal (Normal)	Normal (Normal)	-	Negative
606	124(8.02)115(14.7)	Normal(Normal)	Normal (Normal)	-	Negative

7.3.3.2 Overall assessment of the reproducibility of all qPCR assays using the standard templates developed in chapter six

The biopsy and mucus washing data collected during both clinical studies was used to assess the reproducibility of the template standards used to generate the qPCR assays for the 18S rRNA and *uidA* gene assays in chapter 6, and described in section 7.3.2.6. For both biopsy and mucus washing analysis, templates, in terms of reproducibility of absolute quantities, showed variation from - 4 to + 11 % for 18S rRNA genome templates, and from - 13 to + 8.8 % for the linearised plasmid *uidA* DNA templates. These were all within our accepted defined parameters.

It was not possible to run actual patient biopsy DNA templates in qPCR on more than one occasion due to their use for multiple assays and limited DNA quantity. In the aphthous ulcer study (see section B) there was sometimes only a single biopsy from one anatomical site or feature, this made it more appropriate to assess inter-assay variations based on the known standard templates.

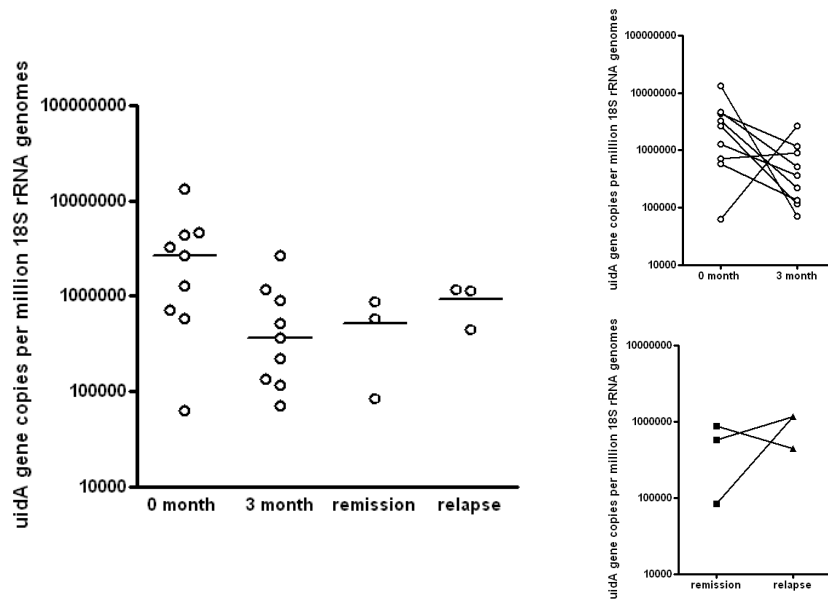
7.3.3.3 Combined qPCR biopsy and mucus washing data for patients in the Plantain clinical trial

Table 7.2 and figure 7.2A show the total *uidA* gene quantities standardised per million 18S rRNA genomes present in the combined biopsies and mucus washings of patients who remained in remission and those who relapsed during the clinical trial for comparison. Figure 7.2A also shows spread and correlation. There was no statistically significant difference between the combined mucus and biopsy *E.coli* quantities of the three relapse patients between remission and relapse periods.

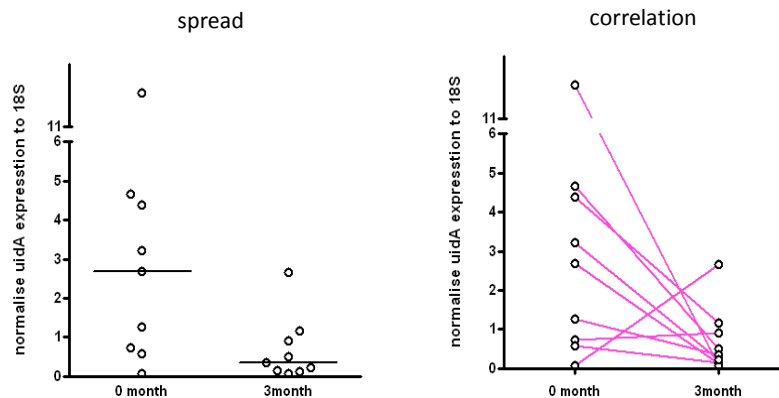
Figure 7.2B shows the isolated data for the patients who remained in remission during the plantain clinical trial. Quantities of *uidA* genes are standardised per million 18 rRNA genomes present in the biopsies of the nine patients in the plantain trial who did not clinically relapse or withdraw during the plantain trial. (i.e 9 out of 12 patients, see table and figure 7.1) Both data spread and correlating pairs are shown in the figure 7.2. The graphs show a significantly lower quantity of *uidA* genes present in these patient biopsies after three months involvement in the trial. Their treatment randomisation to plantain or placebo remains unknown, but this significant finding in patients who remained in remission is interesting.

Table 7.2. Total *uidA* gene copies per million 18S rRNA genomes present in the combined biopsies and mucus washings of patients in the plantain clinical trial

Patient	0 months <i>uidA</i> gene copies/million 18S rRNA genomes	3 months <i>uidA</i> gene copies/million 18S rRNA genomes		Patient	Remission <i>uidA</i> gene copies/million 18S rRNA genomes	Relapse <i>uidA</i> gene copies/million 18S rRNA genomes
201	2684000	115800		101	877500	446350
202	4381000	1152000		502	84300	1149800
601	717900	897600		603	573700	1148700
605	583500	132100				
606	63020	2671000				
504	4673000	509000				
604	1266000	357500				
103	13190000	69310				
503	3217000	219600				



A



Wilcoxon's signed ranks test P = 0.0273

B

Figure 7.2 Quantification of combined biopsy and mucus wash quantities of *uidA* gene copies / million 18S rRNA genomes from the large intestinal tissues of all patients in the biopsy arm of the plantain clinical trial (A), and remission patients alone (B). Both spread and correlation are shown in both figures. Figure B shows the combined standardised mucus and biopsy quantities of *uidA* gene copies from patients who remained in remission at 0 and 3 months. Figures represent the average of 3 - 4 pooled biopsies and washings from the same location with each sample also having PCR performed in triplicate for each patient. (N3-4, n9-12, median is shown). Wilcoxon's two sided signed rank sum test is significant for lower 3 month *uidA* quantities versus month 0 *uidA* quantities following randomised treatment. For all data shown , '0' and '3 months' refers to quantification timepoints for those patients in remission throughout, whilst the panels labelled 'remission' and 'relapse' refers to quantification timepoints in the three patients who relapsed clinically (for exact relapse time see appendix five, and figure 7.1).

7.3.3.4 *uidA* qPCR data for biopsies taken from relapse patients in the plantain clinical trial

uidA gene quantities from paired biopsy samples for the three patients who relapsed are shown in table 7.3, and figures 7.3 and 7.4. The data are expressed per million 18S rRNA genomes (figure 7.3) and per mg (figure 7.4) for comparison. Figure 7.3 shows a 3 fold increase in *uidA* genes per million 18S rRNA genomes when patient 603 relapsed, though patient 101 experienced a 3 fold decrease in *uidA* gene quantity, with patient 502 equivocal, having relapse *uidA* genes 83% of remission quantities.

When data were analysed by *uidA* genes per mg (figure 7.4), the same trends were seen though absolute numbers were different. The biopsies from patients 101 and 502 had fifty one fold and five fold lower quantities of *uidA* genes respectively in relapse specimens, and similarly the *uidA* quantities in the relapse biopsies from patient 603 were eight fold elevated. This lends some support to the use of 18S rRNA genomes as a denominator because the trends in *uidA* quantity were similar to those produced on an analysis of *uidA* quantities per mg, offsetting concerns that inflammatory human cells, which contain the 18S rRNA gene, might distort an accurate denominator for biopsy size.

Table 7.3 *uidA* gene copies per million 18S rRNA genomes and per mg large intestinal tissue biopsy for the three patients who relapsed during the biopsy arm of the plantain trial. Figures are averages of 3-4 biopsies from the same location, with PCR performed in triplicate for each biopsy following DNA extraction.

	Remission	Relapse	Remission	Relapse
Patient number	<i>uidA</i> gene copies / million 18S rRNA genomes	<i>uidA</i> gene copies / million 18S rRNA genomes	<i>uidA</i> gene copies/mg biopsy tissue	<i>uidA</i> gene copies/mg biopsy tissue
101	17500	5350	228	4.49
502	59800	49800	350	72
603	22700	61700	51.9	419

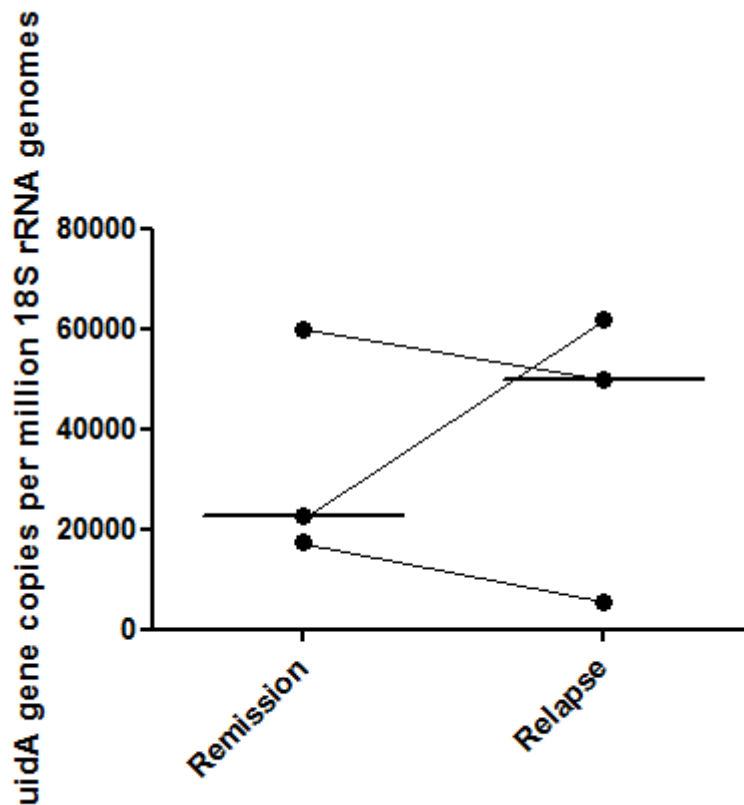


Figure 7.3 *uidA* gene copies per million 18S rRNA genomes for the three patients who relapsed during the biopsy arm of the plantain trial. Data points are averages of 3-4 biopsies from the same location, with PCR performed in triplicate for each biopsy. Patient remission and relapse samples are connected, and median is shown. There is approximately a 3 fold increase in gene copies for patient 603, and 3 fold decrease in *uidA* gene copies for patient 101. Analysis of data using Wilcoxon's two sided signed rank sum test reveals no significant differences between remission and relapse data, though statistical analysis is not really appropriate for $n = 3$ patients.

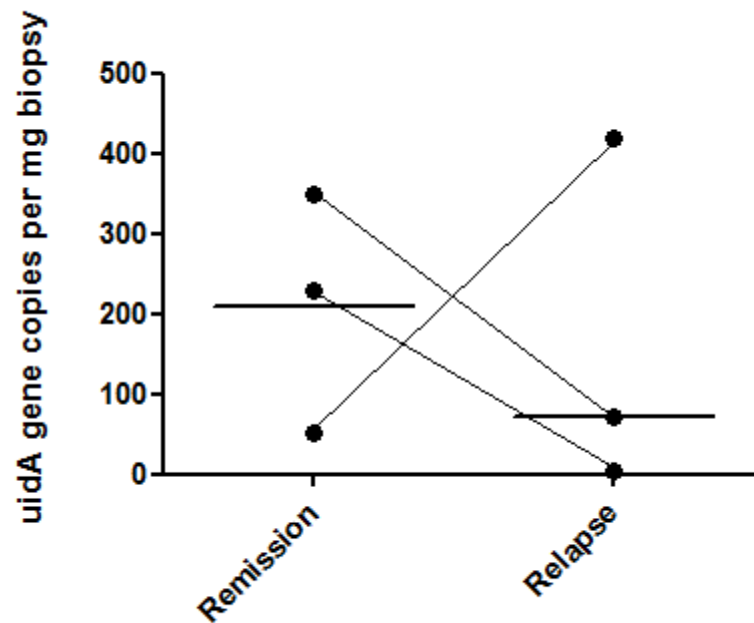


Figure 7.4 *uidA* gene copies per mg large intestinal tissue for the three patients who relapsed during the biopsy arm of the plantain trial. Dots are averages of 3-4 biopsies from the same location, with PCR performed in triplicate for each biopsy for each patient following DNA extraction. Patient remission and relapse samples are connected, and median is shown. There are 5 fold and 51 fold decreases in *uidA* gene copies in patients 502 and 101 respectively when they relapse, but an 8 fold increase in *uidA* gene copies for patient 603. Analysis of data using Wilcoxon's two sided signed rank sum test reveals no significant differences between remission and relapse data, though statistical analysis is not really appropriate for $n = 3$ patients.

7.3.3.5 qPCR mucosal washings data for patients who relapsed during the plantain clinical trial

The mucosal washings from the biopsies of patients who had relapsed were analysed by qPCR and the data are shown in table 7.4 and figure 7.5. The washings from patients 603 and 101 showed a 2 fold increase and 2 fold decrease respectively, in the amount of *uidA* genes quantified per million 18S rRNA genomes, which corresponds to the findings seen with trends in the biopsy specimens from these patients. A disparity was seen in the washings from patient 502, a forty five fold increase was seen in the *uidA* gene quantites per million 18S rRNA genomes, whereas in the corresponding biopsy tissues a fall in numbers was seen. The bacterial species present in mucosal washings, however, may be very different to those on or in the biopsy (see introduction chapter 2), and a direct correlation is not necessarily expected.

Table 7.4 *uidA* gene copies per million 18S rRNA genomes for mucosal washings from the three patients who relapsed during the biopsy arm of the plantain trial.

Figures are an average of the washings from the 3-4 biopsies from the same location, with PCR performed in triplicate for each biopsy following DNA extraction (N3-4, n9-12).

Patient	Remission <i>uidA</i> gene copies/million 18S genomes	Relapse <i>uidA</i> gene copies/million 18S rRNA genomes
101	860000	441000
502	24500	1100000
603	551000	1087000

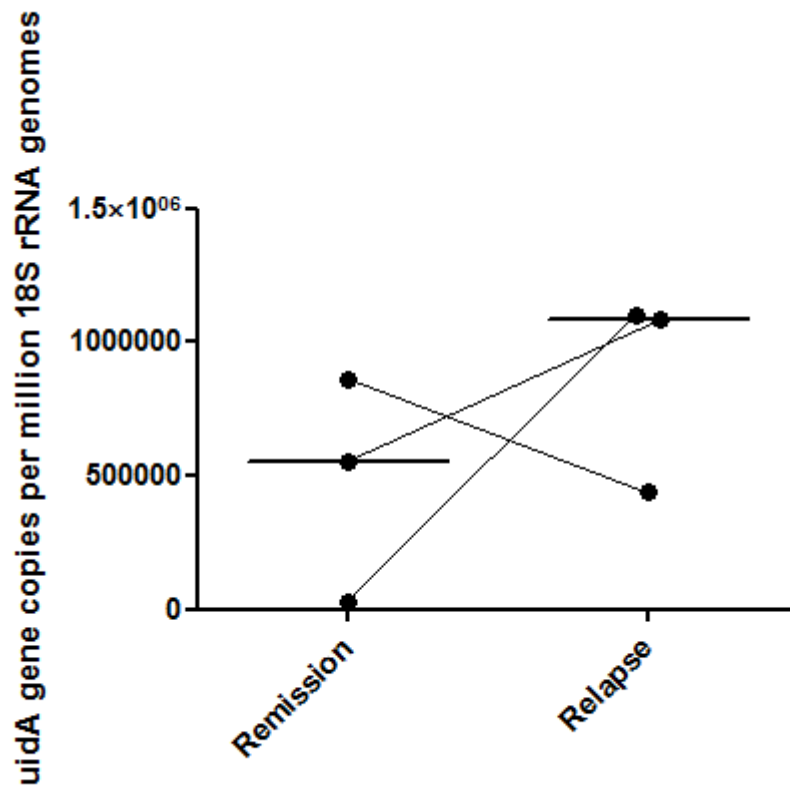


Fig 7.5 *uidA* gene copies per million 18S rRNA genomes for mucosal washings from the three patients who relapsed during the biopsy arm of the plantain trial.

Figures are an average of the pooled washings from the 3-4 biopsies from the same location, with PCR performed in triplicate for each biopsy following DNA extraction (N3-4, n9-12) for each patient. Patient remission and relapse samples are connected, and median is shown. There is a 2-fold increase in gene copies from the mucus washings of patient 603, and a 2-fold decrease in *uidA* gene copies from the mucus washings from patient 101 following relapse. There is a 45-fold increase in the *uidA* gene copies from mucus washings in patient 502 when they relapsed. Analysis of data using Wilcoxon's two sided signed rank sum test reveals no significant differences between remission and relapse data.

7.3.4 Conclusions

- 1) The assay worked well to quantify the bacteria in biopsies and mucus washings from clinical trial samples of large intestinal tissue.
- 2) There was a significantly lower quantity of *E.coli* bacteria in the combined mucus washings and biopsies from patients who were in clinical remission at three months compared to the same individuals (also in remission) at the start of the trial period. Although the data are blinded this implies a reduction in mucosa-associated bacteria with the trial intervention (plantain fibre or maltodextrin placebo).
- 3) It was not possible to demonstrate a statistically significant difference in *E. coli* bacteria present in the biopsies of patients ($n = 3$) who relapsed clinically, compared to the bacteria present in the same patient during their period of remission but further studies are ongoing.

7.4 Section B. The quantification of *E. coli* in aphthous ulcers

7.4.1 Introduction. Aphthous ulcers as the earliest lesions in active CD

Although attempts have been made, aphthoid or aphthous ulcers are difficult to define in macroscopic or microscopic terms. In a previous study surface ‘erosions’ identified by a ‘red halo’ appearance on white light microscopic examination of lymphoid tissue biopsies collected after magnifying colonoscopy were 200 µm in diameter (324). Radiographically they appear as a fleck of barium surrounded by a translucent halo (325), and they are generally regarded as 2-5 mm in diameter. For the purpose of this thesis, I have pragmatically defined an aphthous ulcer as an ulcer lesion of 4 mm or less, as estimated by the fully open standard biopsy forceps. An aphthous ulcer is the earliest lesion seen in active CD, corresponding to disease activity and also the earliest lesion seen in recurrence of CD after operative resection (326), and often seen at the site of the anastomosis within 3-18 months, usually preceding deeper ulceration or formation of granulomata (327). This type of lesion is not wholly specific to CD because it can also be seen in ulcerative colitis and diversion colitis in extreme cases (328).

The literature on colorectal and ileal aphthous ulcers is sparse, although the aphthoid ulcers seen in the buccal cavity have been much more thoroughly researched (329-331). Oral ulcers can also be a common manifestation of IBD (332). To date, there have been no in depth studies characterising the colorectal microbiota occupying the aphthous ulcer niche, and the nature of the colonising bacteria is poorly

understood. Bacteria including *E. coli*, seen in inflamed CD tissue in periods of disease activity, are often increased (88), and it seems probable this would be the case too for aphthoid ulcers and lymphoid follicles particularly as it has been observed that the earliest lesions seen in CD are seen at the ileo-caecum (27), where the Peyer's patches containing M cells are present. The increase in numbers of *E. coli* may be a primary phenomenon or secondary to mucosal inflammation. In this work qPCR was used to quantify and compare numbers of *E. coli* within aphthous ulcer and ileal tissue, including the lymphoid follicles of Peyer's patches, with control or non-inflamed tissue, which would be expected to have smaller amounts.

To evaluate CD and control tissues using the bacterial quantification qPCR methods developed in Chapter six, protocols for recruitment of patients to investigate our hypotheses were designed. The results of the biopsy quantification of *E.coli* bacterial numbers were assessed alongside endoscopic, histological, and clinical parameters. This clinical study is explained in more detail in method section 7.4.2.1 - 7.4.2.2.

7.4.2 Methods

7.4.2.1 Patients

Patients were recruited from the endoscopy lists performed routinely at the Royal Liverpool and Broadgreen University Hospitals NHS Trust. Requests for colonoscopy with or without ileoscopy were reviewed on a weekly basis, and potentially suitable patients who satisfied the inclusion and exclusion criteria (7.4.2.2) for CD patients

and controls were invited to participate in the study by letter at least 48 h in advance. The aim was to try and identify patients who potentially had a new diagnosis of CD, or who had active disease or recurrence, in order to maximise detection of aphthous ulcer numbers. Appropriate patients were recruited following full informed consent. The study had full local ethical approval (Northwest 2- Liverpool Central, reference 10/H1005/25) and NHS Trust approval number 3945.

The clinical data collected for each patient are summarised in Appendix One, including indication for the procedure, current medication, bowel preparation used, site of disease, disease activity evaluated using the Harvey Bradshaw Index score (for CD patients only (333)), co-morbidities and recent C-reactive protein (CRP) measurement.

Five patients were recruited (3 CD patients and 2 healthy controls, as described in appendix one), and samples taken as described in section 7.4.2.3.

7.4.2.2 Inclusion and exclusion criteria

The inclusion criteria for CD patients were as follows:

- 1) Patients aged 18 years or over with CD diagnosed by conventional clinical, radiological and histological criteria.
- 2) CD involving small bowel, colon or both.
- 3) Patients with CD disease undergoing colonoscopy for routine clinical investigation (reasons for this typically included assessment of disease extent, assessment of possible post-operative relapse, or surveillance for colon cancer)

The inclusion criteria for controls were:

- 1) Patients undergoing routine colonoscopy for other reasons, for example colonic symptoms such as rectal bleeding, diarrhoea and change in bowel habit, assessment of diverticular disease, and sporadic cancer surveillance.

The exclusion criteria applied to both groups were:

- 1) Patients aged under 18 years or unable to give informed consent.
- 2) Any antibiotic use within the previous 4 weeks
- 3) Any serious intercurrent infection.
- 4) Warfarinised patients and patients on anti-platelet drugs

7.4.2.3 Sampling procedure for intestinal samples and evaluation of the macroscopic appearance of colonic and ileal mucosa

The methods used for macroscopic assessment, sampling and histological examination of colonic tissue are as described in section 7.3.2.3 and 7.3.2.4, with the modifications that for the aphthous ulcer study patients received either Klean-Prep or Moviprep® 24 hours prior to colonoscopy with or without ileoscopy. On the day of the procedure up to twelve research biopsies were taken from small and large intestinal locations in each patient in a single colonoscopy session in addition to those clinically indicated. Particular priority was given to sampling aphthous ulcers and lymphoid follicles when seen.

The macroscopic appearance at endoscopy was documented (see Appendix two), for later comparison with microscopic appearance and *uidA* gene quantification from biopsies at the same region of the gastro-intestinal tract. For the aphthous ulcer study, the entire colon and ileum was examined as indicated, using a standard Olympus endoscope with biopsies taken at sites of pathology and from control tissue with standard disposable endoscopic forceps.

For results from routine histological assessment of tissue see Appendix Two. Laboratory processing of samples to extract DNA, and perform qPCR is described in sections 7.3.2.5, with assay reproducibility assessed as per section 7.3.2.6. For the aphthous ulcer trial a triplicate qPCR quantification of a single biopsy sample was performed, with human cell numbers again quantified and *uidA* gene copies expressed relative to one million 18S rRNA genome copies as well as per mg of biopsy tissue (wet mass).

7.4.3 Results

7.4.3.1 Clinical, macroscopic and histological information

Five patients were recruited, 3 CD patients and 2 healthy controls. One CD patient had colonic, and two had ileo-colonic disease respectively (for patients coded from CD1 – CD 3, with CD 3 also having had a previous right hemicolectomy). Their basic clinical characteristics are outlined in Appendix One. All patients presented with either abdominal pain, diarrhoea, or for disease surveillance, with control HBI index scores between 0-5 (CRP for both was <5, or normal) and CD patients had HBI index

scores between 4 and 13, with CRP's between 9 and 37. Two out of three CD patients were on maintenance 5-amino salicylic acid (5-ASA) compounds, with the third patient having a new diagnosis. One of these patients was also on steroids (prednisolone) for resistant asthma rather than for gastroenterological reasons.

The findings from endoscopy are outlined in Appendix Two, the only significant macroscopic inflammatory changes are seen with patients coded CD1 and CD2. CD1 patient had linear CD scars, CD2 patient had active disease with multiple aphthous ulcers in the terminal ileum with splenic flexure ulceration as well as rectal inflammation and a new diagnosis of CD was made. Of the control patients CONT 1 had a descending colon polyp, and CONT 2 had a normal appearance macroscopically.

Histologically, patients CD2 and CD3 had ileitis, with CD2 also showing associated colitis and granulomata in both ileum and colon, confirming the diagnosis made endoscopically. Tissue from CONT 1 confirmed tubulovillous adenoma with histological samples from CONT 2 having normal appearance. Full histological details are in Appendix Two.

7.4.3.2 Physical details of biopsies taken

Physical data of the biopsies taken, including average mass and DNA quantity extracted are outlined in Appendix Three. The amount of DNA extracted from the mucus washings of these biopsies was also quantified.

7.4.3.3 Assessment of *uidA* gene quantity in small and large intestinal tissue

The results demonstrating qPCR quantification of *uidA* genes in small intestinal biopsy tissue are shown in figure 7.6 and table 7.5. *uidA* gene quantities expressed per mg small intestinal tissue are presented in table 7.6 and figure 7.7.

Tables 7.7, figure 7.8 and table 7.8, figure 7.9 illustrate the results obtained for *uidA* gene quantification in large intestinal biopsies per million 18S rRNA genomes and per mg biopsy tissue respectively.

Table 7.5 *uidA* gene copy numbers per 1×10^6 18S rRNA genomes in small intestinal samples TI = terminal ileum, AU = aphthous ulcer, B = biopsy code no, LF = lymphoid follicle, SI = small intestine, serp = serpingous. ND = not detected

SMALL INTESTINAL TISSUE – QUANTIFICATION OF E .COLI -ANALYSIS PER 1×10^6 18S rRNA GENOMES			
CONTROL TISSUE	<i>uidA</i> gene copies / 1×10^6 18S rRNA genomes	CD TISSUE	<i>uidA</i> gene copies / 1×10^6 18S rRNA genomes
CONT 2 TI	541000	CD1 B6 TI	ND
CONT 2 LF	413000	CD2 SERP ULCER TI	ND
		CD2 AU	11600
		CD3 TI B	109000
		CD3 SI	96100
		CD3 TI B	2060000
		CD1 B5 LF	ND

Table 7.6 *uidA* gene copies per mg small intestinal samples TI = terminal ileum, AU = aphthous ulcer, B = biopsy code no, LF = lymphoid follicle, SI = small intestine, serp = serpingous. ND = not detected

SMALL INTESTINAL TISSUE - QUANTIFICATION OF E.COLI ANALYSIS PER MG			
CONTROL TISSUE	<i>uidA</i> gene copies/mg tissue	CD TISSUE	<i>uidA</i> gene copies/mg tissue
CONT 2 TI	108	CD1 B6 TI	ND
CONT 2 LF	1650	CD2 SERP ULCER TI	ND
		CD2 AU	157
		CD3 TI B	86.5
		CD3 SI	60.7
		CD3 TI B	187
		CD1 B5 LF	ND

Table 7.7 *uidA* gene copies per 1x10⁶ 18S rRNA genomes in large intestinal samples. AC = ascending colon, SC = sigmoid colon, SF = splenic flexure, LI = non specific large intestinal sample, B = non specific colon sample. ND = not detected

LARGE INTESTINAL TISSUE - QUANTIFICATION OF E.COLI - ANALYSIS PER 1X10⁶ 18S rRNA GENOMES			
CONTROL TISSUE	<i>uidA</i> gene copies / million 18S rRNA genomes	CD TISSUE	<i>uidA</i> gene copies / million 18S rRNA genomes
CONT1 B1	2470	CD1 B SF	4720
CONT1 B2	29500	CD1 B2 ?	ND
CONT1 B3	ND	CD1 B3 SC	ND
CONT1 B4	7940	CD1 B4 TC	ND
CONT1 B5	33300	CD2 B SF	ND
CONT1 B6	26700	CD2 B SF ULCER	ND
CONT1 B7	4230	CD3 LI	3110000
CONT1 B8	45500	CD3 LI	2020000
CONT2 AC	873000	CD3 LI	187000
CONT2 AC	1090000	CD3 LI	67200
CONT2 SC	2880000		
CONT2 SC	223000		
CONT2 SC	74000		
CONT2 CAECUM	1570000		

Table 7.8 *uidA* gene copies per mg large intestinal tissue AC = ascending colon,

SC = sigmoid colon, SF = splenic flexure, LI = non specific large intestinal sample, B

= non specific colon sample

ND = not detected

QUANTIFICATION OF E.COLI LARGE INTESTINAL TISSUE - ANALYSIS / MG			
CONTROL TISSUE	<i>uidA</i> gene copies / mg tissue	CD TISSUE	<i>uidA</i> gene copies / mg tissue
CONT1 B1	15.3	CD1 B SF	37.6
CONT1 B2	491	CD1 B2	ND
CONT1 B3	ND	CD1 B3 SC	ND
CONT1 B4	11.6	CD1 B4 TC	ND
CONT1 B5	170	CD2 B SF	ND
CONT1 B6	200	CD2 B SF ULCER	ND
CONT1 B7	36.1	CD3 LI	702
CONT1 B8	91.9	CD3 LI	107
CONT 2 AC	125	CD3 LI	72.2
CONT 2 AC	82.1	CD3 LI	172
CONT 2 SC	111		
CONT 2 SC	79.1		
CONT 2 SC	132		
CONT2 CAECUM	49.9		

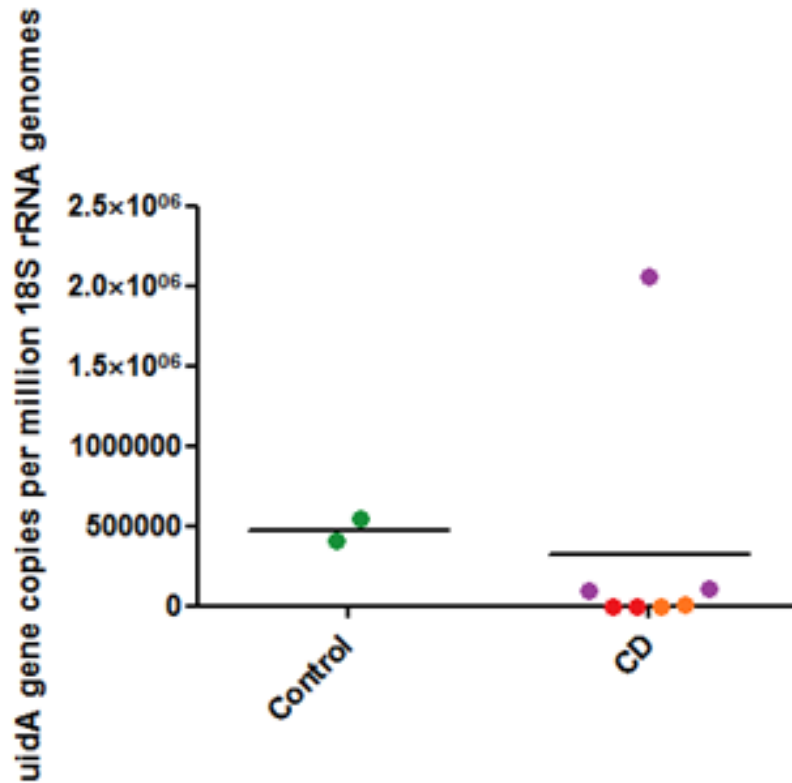


Figure 7.6. A comparison of the *uidA* gene copy numbers seen in small intestinal tissue in control and CD tissue. *uidA* gene copies are expressed per million 18S rRNA genomes, and each dot represents the average of triplicate PCR samples from a single biopsy. The median for the CD group and the control group is shown as a horizontal line. The high outlier represents a terminal ileal biopsy from patient CD3. Two sided Mann-Whitney U test did not show a significant difference between *E. coli* quantities in control vs CD tissue. Samples from a given patient are colour coded: Cont 2 (Green), CD1 (Red), CD2 (Orange), CD3 (Purple). The location each biopsy is from is given in table 7.5.

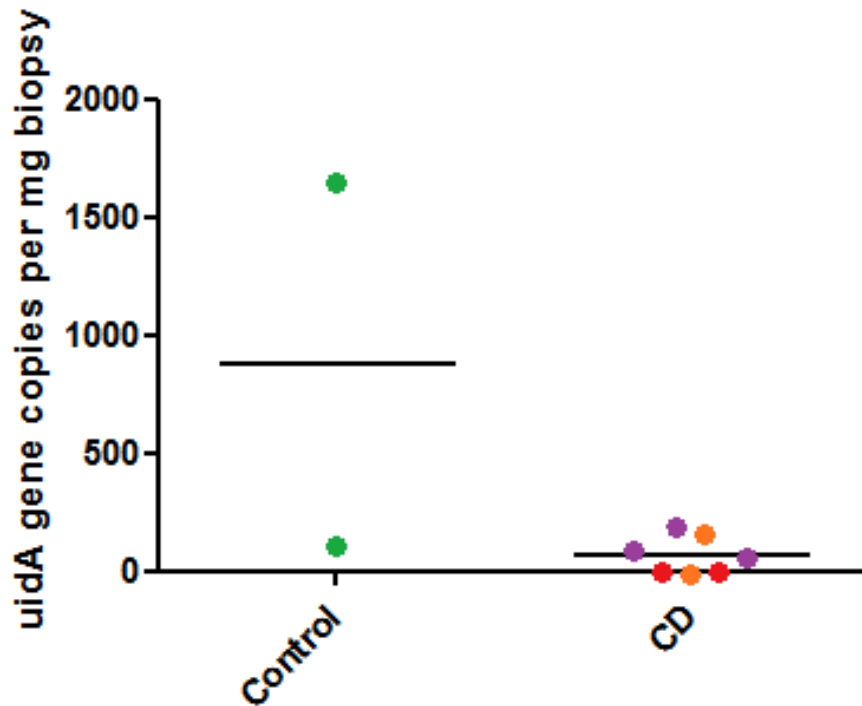


Figure 7.7. A comparison of the *uidA* gene copy numbers seen in small intestinal tissue in control and CD tissue. *uidA* genes are expressed per mg tissue, and each dot represents the average of triplicate PCR samples from a single biopsy. The median for the CD group and the control group is shown as a horizontal line. The high outlier represents a lymphoid follicle biopsy from a control patient. Two sided Mann-Whitney U test did not show a significant difference between *E. coli* quantities in control vs CD tissue. Samples from a given patient are colour coded: Cont 2 (Green), CD1 (Red), CD2 (Orange), CD3 (Purple). The location each biopsy is from is given in table 7.6.

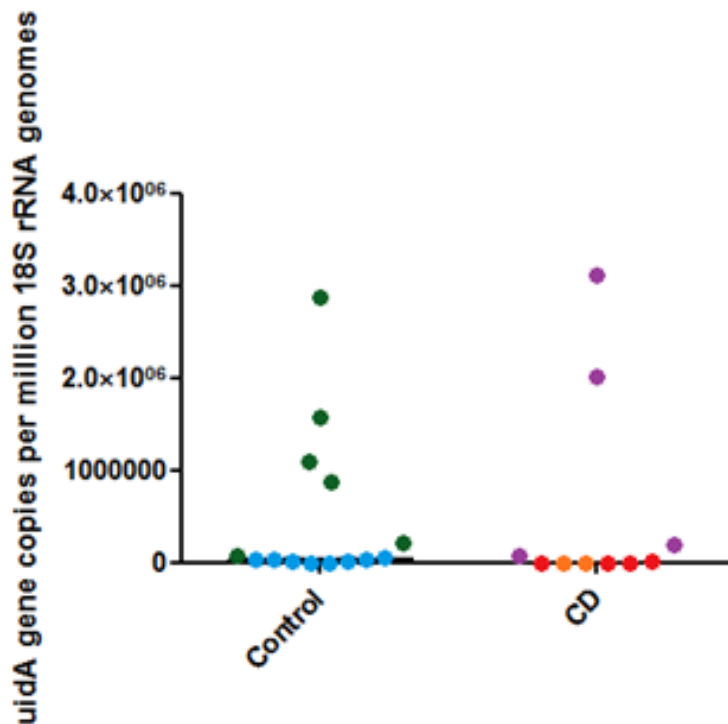


Figure 7.8 A comparison of the *uidA* gene copy numbers seen in large intestinal tissue in control and CD tissue. *uidA* genes are expressed per million 18S rRNA genomes and each dot represents the average of triplicate PCR samples from a single biopsy. The median for the CD group and the control group is shown as a horizontal line. The highest two values were from sigmoid colon biopsies in CD patients, but in controls the highest two values were from ascending colon and caecum. Two sided Mann-Whitney U test did not show a significant difference between *E. coli* quantities in control vs CD tissue. Samples from a given patient are colour coded: Cont 1(Blue), Cont 2 (Green), CD1 (Red), CD2 (Orange), CD3 (Purple). The location each biopsy is from is given in table 7.7.

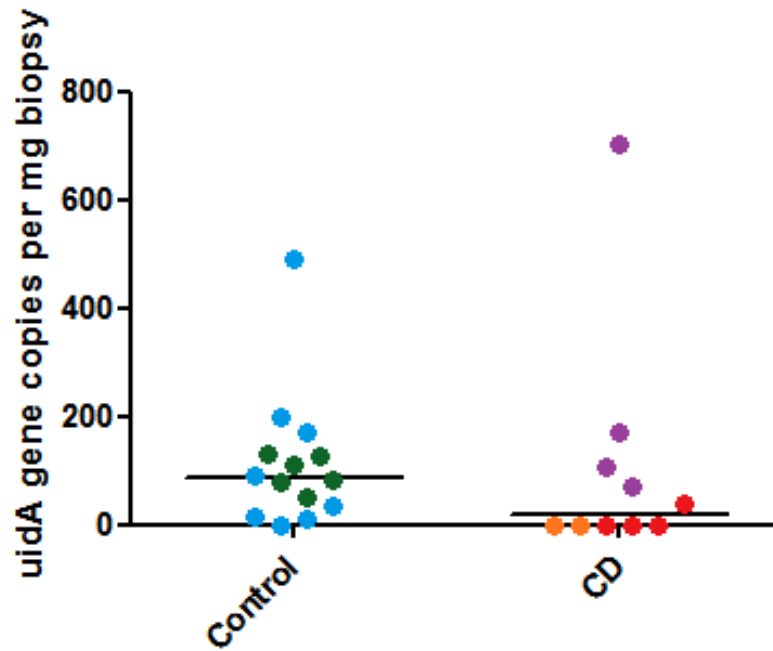


Figure 7.9 A comparison of the *uidA* gene copy numbers seen in large intestinal tissue in control and CD tissue. *uidA* genes are expressed per mg large intestinal tissue and each dot represents the average of triplicate PCR samples from a single biopsy. The median for the CD group and the control group is shown as a horizontal line. The highest two values were from sigmoid colon biopsies in CD and control patients. Two sided Mann-Whitney U test did not show a significant difference between *E. coli* quantities in control vs CD tissue. Samples from a given patient are colour coded: Cont 1(Blue), Cont 2 (Green), CD1 (Red), CD2 (Orange), CD3 (Purple). The location each biopsy is from is given in table 7.8.

7.4.3.4 Mucosal wash data for the aphthous ulcer study

The DNA quantities extracted from the mucosal washings of large and small intestinal biopsies in the aphthous ulcer study are detailed in Appendix four. The highest extracted concentration of DNA was seen in the washing from a biopsy taken from

the site of an aphthous ulcer in the patient newly diagnosed with active CD (patient 2); the DNA quantity was a peak for the small intestinal tissue examined at 90.2ng per μ l, though this did not translate as an increase in *uida* gene quantity per million 18S rRNA genomes (see figure 7.10).

qPCR was also performed on the washings from large intestinal biopsy samples with the scatterplot produced by examining *uida* genes per million 18S rRNA genomes shown in figure 7.11.

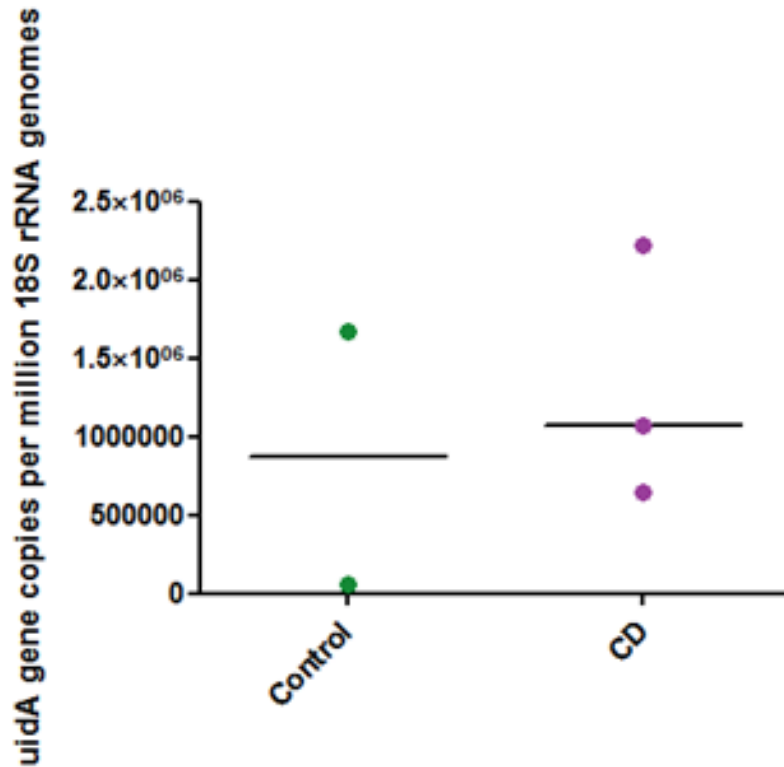


Figure 7.10 Comparison of the *uidA* gene copy numbers seen in small intestinal tissue mucus washings from control and CD tissue. *uidA* genes are expressed per million 18S rRNA genomes, and each dot represents the average of triplicate PCR samples from pooled mucus washings from a single location. Two sided Mann-Whitney U test did not show a significant difference between *E. coli* quantities in control vs CD tissue ($P = 0.8$) but n numbers are of course very small. The median for the CD group and the control group is shown as a horizontal line. Samples from a given patient are colour coded: Cont 2 (Green), CD3 (Purple).

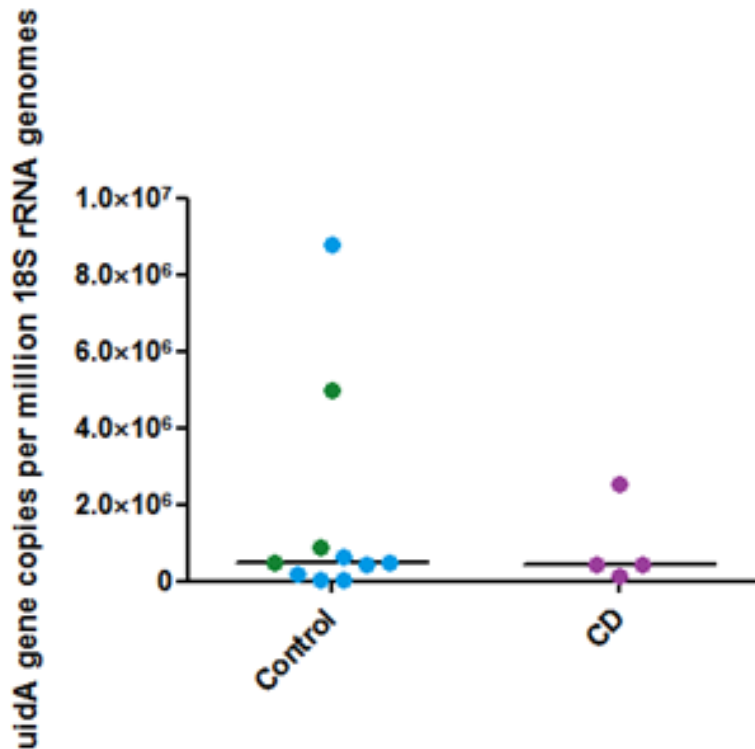


Figure 7.11 comparison of the *uidA* gene copy numbers seen in large intestinal tissue mucus washings from control and CD tissue. *uidA* genes are expressed per million 18S rRNA genomes, and each dot represents the average of triplicate PCR samples from pooled mucus washings from a single location. The median for the CD group and the control group is shown as a horizontal line. Two sided Mann-Whitney U test did not show a significant difference between *E. coli* quantities for control vs CD tissue (P 0.84). Samples from a given patient are colour coded: Cont 1(Blue), Cont 2 (Green), CD3 (Purple).

7.4.4 Conclusions

1) The results to date do not support the hypothesis of increased *E. coli* quantities in aphthous ulcer tissue, or within small intestinal tissue in CD patients compared with controls. However these results are based on small numbers which are inadequate to allow statistical comparison, or to allow comparison between ulcer and non ulcer tissue within the CD patient tissue group.

2) (i) The assay is working well for quantification of *E.coli* on endoscopic biopsy samples from small and large intestine.

(ii) Patients who are negative for *E. coli* at one site are negative throughout the intestine.

3) The assay works well to quantify the bacteria in mucus washings from endoscopic biopsy samples of small and large intestine.

7.5 Discussion

The principal aim of the work in this chapter was achieved when the qPCR assays developed in chapter six were successfully utilised to quantify *uidA* genes in biopsies and mucosal washings from different types of CD tissue, in both periods of disease remission and relapse over time and also in small and large intestinal tissue at the same timepoint. This was done in a robust and reproducible way. It is conceivable that quantification of *uidA* genes in tissue could be used at different stages of CD activity and to assess responses to pharmacological treatments as well as potentially a prognostic indicator of disease progress.

Though each study involved small numbers of patients, there were interesting findings in both. Primarily there was a significantly lower quantity of *E.coli* bacteria in the combined mucus washings and biopsies from patients who were in clinical remission at three months compared to the same individuals who were also in remission at the start of the trial period. Although the data are blinded this implies a reduction in mucosa-associated bacteria with the trial intervention (plantain fibre or maltodextrin placebo), and also does not refute the principal hypothesis that increased *E.coli* bacterial quantities may be associated with CD disease activity; conversely lower *E. coli* quantities were associated with patients being in sustained remission. It will be even more informative when the clinical trial is unblinded and the effect of plantain fibre on both clinical relapse, and *uidA* gene quantity can be assessed in more detail.

Examining the *uidA* gene quantities by qPCR for the three relapse patients showed mixed results. Mucus washings tended to show increased *uidA* gene quantities in relapse even though examination of relapse biopsies produced the opposite trend.

Small numbers were involved, too small to demonstrate a statistically significant difference in standardised *E.coli* numbers between tissues during remission and relapse. The finding of lower DNA quantities in sigmoid biopsies from relapsing patients is not straightforward to explain, but may be associated with lower bacterial DNA, particularly reduced diversity associated with dysbiosis (47).

The numbers of patients recruited in the aphthous ulcer study were clearly too small to demonstrate statistically significant differences in *uidA* gene quantities between control and CD tissue, or to evaluate differences between ulcer and non ulcer tissue in CD patients. This applied for both small and large intestinal tissues, and it is probable that the number of patients recruited needs to be increased in order to detect any differences if they exist. There is further ongoing work to increase the patient number and biopsy sample size and recruit further patients based on the approved protocol and these increased numbers may show statistically significant discernible differences in the microbiota found in control and CD tissue, particularly aphthous ulcers.

There were points of interest. *uidA* quantities in small intestinal tissue of CD and control patients demonstrated a peak number of *uidA* genes in the terminal ileal biopsy from the CD3 patient. This patient had a macroscopically normal ileum, but microscopically had features of ileitis; the identification of high *uidA* gene quantities in the terminal ileal biopsy of a patient with a macroscopically normal ileum but histological ileitis, and some clinical markers of disease activity (HBI of 13 and CRP of 37) raises the possibility of whether bacterial *uidA* qPCR may eventually be used as an accessory predictive tool in pathology. The high quantities of *uidA* gene copies noted in a lymphoid follicle (control patient 2) also suggests a possible role for these sorts of assays in the study of pathophysiological processes, given that this is the key

tissue in the small and large intestine involved in pathophysiological bacterial translocation (125).

Quantification of *E. coli* from the mucosal washes of biopsy tissue from CD and control patient tissue also showed points of interest. The highest extracted concentration of DNA was seen in the washing from a biopsy taken from the site of an aphthous ulcer in the patient newly diagnosed with active CD (CD patient 2); the DNA quantity was a peak for the small intestinal tissue examined at 90.2ng per μ l, though this did not translate as an increase in *uidA* gene quantity per million 18S rRNA genomes. This suggests that the DNA extracted at this site was either from other bacterial species, or human cells in the mucus washings from the biopsy. As the washings were taken from a biopsy with low mass, a source of bacterial DNA other than *E.coli* seems likely, and may reflect colonisation of the aphthous ulcer site.

uidA quantities were highest in both ascending colon, sigmoid colon and caecum in the control patient 2 for both the denominator methods used. High *uidA* level in the in sigmoid colon biopsies from both CD and the control patients, coupled with the findings from qPCR performed on washings from large intestinal biopsy samples confirmed expected findings from what is known about bowel bacterial colonisation numbers in the small and large intestine (334), though again patient numbers were small. Also, the finding that patients who are negative for *E. coli* at one site are negative throughout the intestine, is in accord with a recent study (226).

The use of qPCR to quantify *uidA* genes, and by proxy *E. coli* quantities in CD may become a powerful tool, and perhaps could be used to quantify *E. coli* in clinical trials of antibiotics, as well as to measure the effects of steroids, immunomodulators, and anti-TNF agents on the quantities of *E. coli* in CD. The development of primers for qPCR targeting genes present in ever more specific groups of *E. coli* involved in

CD pathogenesis, for example the long polar fimbriae (*lpf*) gene, in AIEC (335), may refine the use of the qPCR technique yet further.

In terms of choice of denominator for *E.coli* quantification, when data were analysed by quantifying *uidA* genes per mg or per million 18S rRNA genomes as in (218), the same trends were seen, particularly when relapse patient data were examined though absolute numbers were different. This lends some support to the use of 18S rRNA genomes as a denominator. Initially there was a concern that inflammatory human cells, present in areas of intestinal inflammation might produce an overestimation of total cell numbers, with corresponding underestimation of microbial density, particularly in inflamed tissue, which would complicate assessment of diseases such as Crohn's. The use of mass as a denominator would not be distorted by the presence of inflammatory cells per se, but could also be affected by the inflammatory process. The use of both denominators together showing similar trends was a strength of this work.

CHAPTER EIGHT : THE EFFECT OF ANTIBIOTICS AND IMMUNOMODULATORS ON *E. COLI* REPLICATION WITHIN MACROPHAGES *IN-VITRO*

8.1 Hypothesis :

Drugs may have efficacy in CD if they can kill *E. coli* within macrophages. These may include antibiotic combinations but also may include some of the drugs that are commonly used in CD maintenance therapy including immunosuppressives.

8.2 Aim :

To evaluate the effect of antibiotics and immunomodulators at clinically relevant concentrations on *in-vitro* replication of Crohn's disease (CD) mucosally-associated *E. coli* within macrophages.

8.3 Introduction

8.3.1 The use of antibiotics in CD

If CD is caused by bacteria then it would be reasonable to expect a therapeutic response to antibiotics. There is however a relative lack of good controlled evidence to show efficacy. Best evidence probably exists for ciprofloxacin, metronidazole and for the non-absorbed antibiotic rifaximin. This evidence has been reviewed in a recent meta-analysis, discussed in section 2.9 (269). It is evident from the diversity of antibiotics selected to treat Crohn's that the target is unclear, both in respect of the type of bacteria (aerobic, anaerobic or a mixture of both), and in respect of their site (systemic infection or bacteria in the faecal stream, extracellular or intracellular). Without a clearly defined target and appropriate use of combination therapy to reduce development of antibiotic resistance, this approach to therapy is unlikely to make much progress. It should also be noted that metronidazole has anti-inflammatory effects, including blockade of neutrophil recruitment (336) and conversely it is also possible that immunosuppressant drugs might have anti-bacterial effects.

Recent studies have identified mucosa-associated *E. coli* as a potential pathogenic factor in CD. Many independent groups, reviewed in (125) have shown that their presence is markedly increased in Crohn's mucosa compared with mucosa from healthy controls. These particular *E. coli* have an adherent, invasive phenotype, hence their designation as AIEC (337), that also includes an ability to replicate within

macrophages (206;208;242;338). Moreover immunohistochemical (88) and qPCR studies (205) have shown *E. coli* within granulomata in CD tissue, with the exemplar ileal AIEC, LF82, having been shown to induce granuloma formation *in vitro* (339). Proof that these *E. coli* have a causative role rather than gaining entry secondary to inflammation, depends on showing remission of disease in response to appropriate therapy targeting these *E. coli* replicating within macrophages. Similar adherent, invasive *E. coli* have also been found in the analogous granulomatous colitis that affects Boxer dogs (340) and responds to antibiotics (341). Choosing an effective and well tolerated combination of antibiotics which minimises risks of bacterial resistance can be difficult, but offers therapeutic alternatives in CD therapy.

8.3.2 Immunomodulators in CD

There is some evidence that the immunosuppressive azathioprine, a drug commonly used to treat CD (151), may have an anti-bacterial effect (286) – Jarnerot and colleagues described an antibiotic effect of resected Crohn's disease tissue sampled at respective surgery from Crohn's disease patients receiving azathioprine. It is notable that azathioprine seems particularly effective when used to treat fistulating perianal disease (342), a complication of Crohn's where bacteria are likely to have a major role. Conversely, prolonged or excessive use of corticosteroids has been shown to substantially increase the risk of serious sepsis in patients with CD (343) and is associated with increased mortality (data from TREAT i.e. The Crohn's Therapy, Resource, Evaluation, and Assessment Tool Registry). Steroid use in CD is discussed in 1.7.2 and the effects of steroids on macrophages are also discussed in section 2.10.2.

This chapter describes studies performed (i) to assess optimal combinations of antibiotics designed to target CD mucosa-associated *E. coli* replicating within macrophages, (ii) to assess the effects of immunosuppressive drugs commonly used in CD on *E. coli* survival and replication in macrophages. The antibiotic studies were performed to aid design of a clinical trial of use of antibiotics to prevent relapse of CD. Whilst our group had already developed antibiotic regimens, used both individually and in combination, and demonstrated *in vitro* efficacy of their ability to kill intra-macrophage Crohn's *E. coli* (242), their clinical use in treatment of Crohn's has been limited by potential drug-drug interactions or toxicity profiles, such as the bone marrow suppressing effect of trimethoprim when co-administered with azathioprine (344), and the effect of metronidazole to produce ethanol intolerance (345). Long term use of metronidazole has also been associated with peripheral neuropathy (346), whilst ciprofloxacin has a number of neurological side effects (347) and causes tendonopathy (348).

The combination of ciprofloxacin and metronidazole had not been evaluated previously *in vitro*, against *E. coli* and, although metronidazole is specific for anaerobic bacteria, there is a theoretical possibility that *E. coli* might be metabolising anaerobically when within macrophage vesicles. All the antibiotics and immunosuppressive agents used were evaluated at clinically relevant concentrations.

8.4 Aims:

1) To assess optimal combinations of antibiotics, designed to target *E. coli* replicating within macrophages at clinically relevant levels.

2) To assess the effects of immunosuppressive drugs commonly used in CD on *E. coli* replication in macrophages, again at clinically relevant levels.

8.5 Methods

8.5.1 Evaluation of Crohn's mucosa-associated *E. coli* replication within macrophages in the absence or presence of antibiotics

Murine macrophages (J774-A1) cultured *in vitro* (described in section 5.1.2) on standard 24 well plates were infected with adherent, invasive Crohn's *E. coli* isolate HM605 for 2 h (at a multiplicity of infection (MOI) at a ratio of two hundred and fifty to one (bacteria to macrophage), to allow bacterial internalisation. This was followed by removal of extracellular bacteria by three successive PBS washes and gentamicin (20 µg/ml) treatment for 1 hour. This was followed by a further five PBS washes and a further gentamicin treatment as described previously (242). Macrophages were then incubated for a further 3 hours in either the presence of antibiotics at C_{\max} , either metronidazole (20 µg/mL) alone, ciprofloxacin (4 µg/mL) alone, or metronidazole in combination with ciprofloxacin, in comparison to DMSO/RPMI media vehicle control (see section 5.1.2). C_{\max} is defined as the peak plasma concentration of a therapeutic agent achievable by a conventional dosing regimen), with doses used as stated in references (242;349;350), Antibiotics were also assessed at 10% C_{\max} .

8.5.2 Examining the effects of immunomodulators on intra-macrophage replication of *E. coli*.

The action of a range of the therapeutics used in the treatment of CD were assessed which included clinically useful concentrations. Drug concentrations used were; azathioprine (AZA, 4.2×10^{-12} to $4.2 \mu\text{M}$, but also initially concentrations up to $210 \mu\text{M}$), 6-mercaptopurine (6-MP, 0.042 to $4.2 \mu\text{M}$), hydrocortisone (HC, 0.06 to $6 \mu\text{M}$), as well as one of the end metabolites of azathioprine, 6-thioguanine (6-TG, at 8.2×10^{-5} - $820 \text{ fmoles}/8 \times 10^8$ macrophage cells). Following pre-incubation of J774-A1 murine macrophages for 24 hours with these drugs, they were infected with Crohn's *E. coli* again as per section 8.5.1.

All immunomodulator dose ranges tested included levels that are of clinical relevance in CD. For azathioprine and 6-MP, $1.05 \mu\text{M}$ and lower (287) were of most relevance as therapeutic concentrations of azathioprine are in the range of up to $2 \mu\text{M}$ (351). For 6-TG, the concentration of interest is 82 fmol per 8×10^8 cells, which is the equivalent of a therapeutic level of the active metabolite 6-TG seen with conventional dosing of patients with azathioprine or 6-MP at $1.5\text{-}2 \text{ mg/kg}$ per day (352-354).

A daily dose of 40 mg prednisolone, an oral steroid commonly used to control IBD relapse is equivalent to a $6 \mu\text{M}$ concentration of Hydrocortisone when used *in vitro* (355).

8.5.3 Quantifying the effects of immunomodulatory agents and antibiotics on *E. coli* replication within macrophages

Following infection, macrophages were lysed (after 6 h) with 1% (v/v) Triton X-100 and internalised bacteria enumerated by overnight growth on LB agar.

The actual number of *E. coli* added on the day of the infection was confirmed by performing serial one in ten subdilutions of the neat bacteria and plating 50µl of these dilutions on standard LB agar with the resulting number counted after overnight incubation at 37°C. These counts were used to produce a correction factor so that numbers could be directly counted inter-experiment.

Data were finally analysed with bacterial numbers present in macrophages treated with experimental antibiotics and/or drugs compared to untreated infected cells at 6 hours. The datasets were statistically analysed using Kruskal-Wallis non-parametric ANOVA followed by post-hoc pairwise comparison of all treatment means (Conover Inman). Differences were considered significant when $P < 0.05$.

8.5.4 Assessment of the effect of therapeutic agents on macrophage growth and viability

To establish whether the differences in treatment on *E. coli* replication within macrophages were due to a real treatment effect rather than secondary to significant differences in toxic effect to the macrophages between untreated and drug treated groups viability and growth were assessed. J774-A1 macrophage growth rate was estimated by direct haemocytometry over three days. On three separate occasions J774-A1 macrophages were seeded into 24 well plates at the same concentration

(1×10^5 /ml with 1 ml added). On day 2 and day 3 a proportion of these wells were used to perform cell counts after cells were carefully removed using cell scrapers with cells counted by direct vision under the microscope/haemocytometer. Cell numbers and growth rate were assessed for untreated cells and for cells pre-treated with the maximum concentration and clinically useful concentrations of hydrocortisone and azathioprine used in the replication assay (8.5.2). Cell numbers in each group were averaged from replicate triplicates on the three occasions and analysed using Kruskal-Wallis ANOVA.

As a further measure to assess J774-A1 macrophage viability after exposure to the pharmacological agents the Toxilight™ adenylate kinase assay kit was used. The principle of the kit is that if a concentration of drug results in greater toxicity, cell damage results in leakage out of the cell, particularly increased leakage of adenylate kinase to the cell culture medium.

Cell supernatants were evaluated as per manufacturers instructions (protocol 2, of kit LT07 – 217), after identical experimental exposures. The macrophage cells had been treated identically to the standard experiments above but toxicity was measured in place of replication for peak doses of therapeutic agents. Adenylate kinase levels produced by RPMI growth media, PBS, and the bacterial *E.coli* suspension alone were used to measure background adenylate kinase release in the absence of treatment and the effect of cell damage caused by bacterial infection of the macrophages alone. Following exposure relative luminescence units were measured.

8.5.5 Assessment of any direct bacteriocidal effect of immunomodulator drugs on bacterial viability outside macrophages

In order to examine whether the drugs used had a direct bacteriocidal effect, independent of modulation of macrophage function, a series of experiments were conducted. *E. coli* HM605 were used at the same bacterial quantity per ml as was used to infect macrophages, but scaled down for use in a 96 well plate. They were inoculated into RPMI media, without antibiotics, containing the immunomodulator drugs used in the macrophage experiment at their peak doses. Hydrocortisone was made up tested at 6 μ M, azathioprine at 210 μ M, ciprofloxacin at C_{\max} 4 μ g/mL, and 6-MP at 4.2 μ M with 6-TG used at the equivalent concentration of 820 fmol per 8×10^8 cells, and drug solutions were pipetted into a 96 well plate in the same ratio as in the macrophage experiment, in a ratio of ten to one, i.e. 18 μ l bacteria added to 180 μ l of drug containing media and these were incubated at 37°C for up to 6 h. A series of replicate wells were set up with identical drug concentration and initial inoculum, and 50 μ l of each well were removed at sequential timepoints. These timepoints were 1,2,3,4,5, and 6 h to mirror the period of time the *E.coli* were exposed to drugs in the intra-macrophage replication experiments, in sections 8.5.1 – 8.5.2. Overall the *E.coli* were exposed to the immunomodulator drugs for 6 h and ciprofloxacin for 3 h. These samples were subdiluted, 10 fold, up to 6 times, in PBS in further 96 well plates to produce countable colonies, and these were plated out on standard LB agar, incubated at 37°C overnight, and enumerated the following day, standardised as CFU /ml.

The actual number of *E. coli* added on the day of the infection was confirmed as per the macrophage assay, to allow inter-experiment comparison (as described in section 8.5.3).

8.6 Results

Table 8.1 C_{\max} of antibiotics used in this thesis

Antibiotic	C_{\max} ($\mu\text{g/ml}$)	Reference
Ciprofloxacin	4	(242)
Metronidazole	20	(350)

8.6.1 The effect of antibiotics on *E. coli* replication within J774-A1 macrophages

When ciprofloxacin was used alone to treat macrophages at C_{\max} , there was a significant inhibition of *E.coli* replication within macrophages. When ciprofloxacin and metronidazole were used in combination to treat macrophages at C_{\max} , there was no statistically significant difference between the use of ciprofloxacin alone, compared to its use in combination with metronidazole. This was perhaps because the level of intra-macrophage killing by ciprofloxacin was so high at C_{\max} that it would be difficult to detect a difference (figure 8.1). So the experiments were repeated again at 10% C_{\max} (figure 8.2). The effect of metronidazole at C_{\max} on *E.coli* replication was not significantly different from the bacterial replication in the untreated macrophage group, so it could be predicted that it would have no effect either at 10% C_{\max} , which

was confirmed (figure 8.2). Similarly at 10% C_{\max} , there was no additional effect in combination with ciprofloxacin seen with metronidazole either (figure 8.2). In fact it inhibited the effect of ciprofloxacin, though not with statistically significant difference between these two treatment groups, though the combination therapies significance value at 10% C_{\max} was $p = 0.07$ relative to untreated macrophages. The level of replication seen within the untreated macrophages was similar to that seen previously (242).

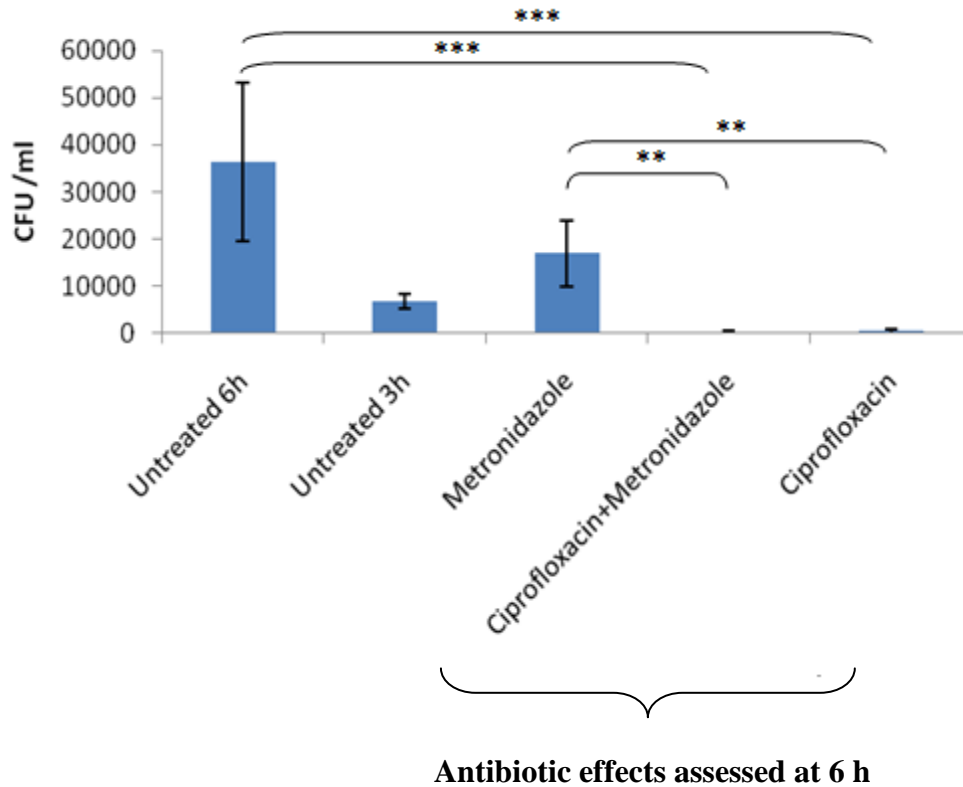


Figure 8.1 The effect of antibiotics used at C_{max} on replication of HM605 *E coli* within J774-A1 macrophages at 6 h. Kruskal Wallis non parametric ANOVA N4, n12, (where N = no of different experimental days, and n = total number of wells used overall per experimental exposure, usually 3 wells per experimental day). ** $p < 0.01$, *** $p < 0.001$ Inhibitory effects are assessed compared with untreated cells at 6 h. Mean CFU/ml \pm SE are shown.

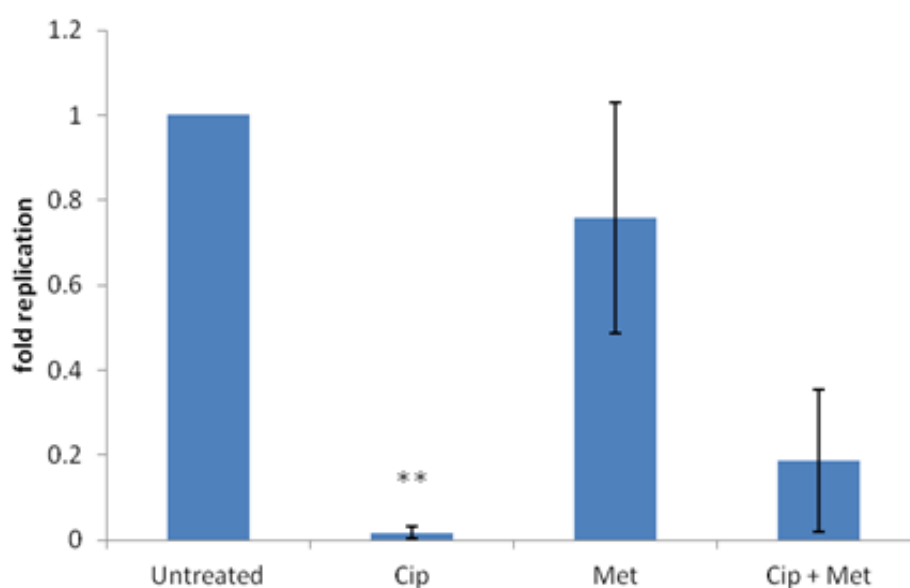


Figure 8.2 Crohn's *E. coli* HM605 replication within J774 murine macrophages at 6 h in the absence or presence of antibiotics used at 10% C_{max}

The effect of both antibiotics used at 10% C_{max} on HM605 *E. coli* replication within J774-A1 macrophages expressed as fold replication relative to untreated macrophages at 6 h. Kruskal Wallis non-parametric ANOVA: ** $p < 0.01$ (N3, n9). Mean fold replication \pm SE is shown.

8.6.2 The effect of immunomodulators on intra-macrophage *E.coli* replication.

The use of supra-therapeutic ($> 2 \mu\text{M}$) concentrations of azathioprine resulted in inhibition of the replication of *E.coli* within macrophages. This was statistically significant at all the doses used apart from the $70 \mu\text{M}$ dose, which showed non statistically significant inhibition (figure 8.3). The inhibitory effect seen with azathioprine was evaluated at therapeutic levels and below, and in fact an inhibitory

effect on intra-macrophage *E.coli* replication was seen at concentrations as low as 4.2×10^{-9} μM , with the effect lost when azathioprine was used at a 1000x lower dilution than this (figure 8.4).

Interestingly there was no significant effect observed on intra-macrophage *E. coli* replication when macrophages were exposed to 6-MP doses at clinically useful concentrations compared to untreated macrophages. Ciprofloxacin was used as a positive control and significantly inhibited *E. coli* replication ($p=0.0054$) within murine macrophages (figure 8.5).

The active metabolite of azathioprine, 6-TG, significantly inhibited the replication of *E.coli* within macrophages in a dose dependent fashion (figure 8.6). The peak suppression of *E.coli* replication was seen at a 6-TG concentration of 82 fmol per 8×10^8 cells, which is the level expected in the blood with a standard dosing regime of azathioprine, and inhibition of replication was maintained at up to 1000-fold lower concentrations. Interestingly a 10 fold higher concentration than the usual therapeutic level produced less suppression of *E. coli* replication though not of statistical significance (figure 8.6).

Hydrocortisone also inhibited intra-macrophage replication of *E.coli* in a dose dependent fashion, which was maximal at a 0.6 μM concentration (figure 8.7). A 6 μM hydrocortisone concentration *in vitro* is approximately equivalent to a conventional IV hydrocortisone dose of 100 mg, or an oral daily prednisolone dose of 40mg (355).

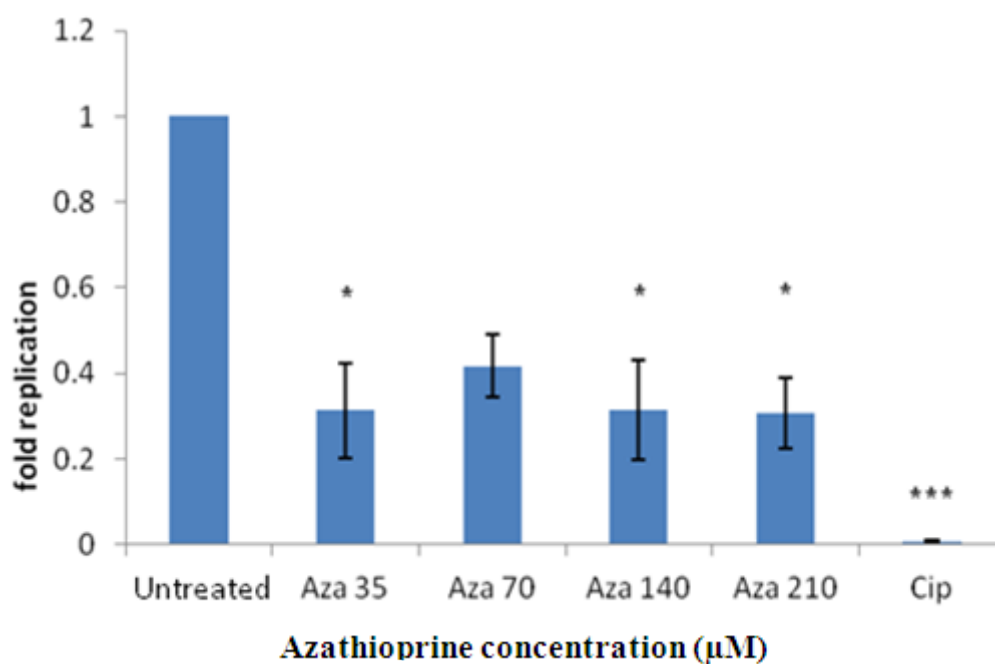


Figure 8.3 The effect of high dose azathioprine on intra-macrophage *E.coli* replication. The effects of pre-incubation of J774-A1 macrophages with high doses of azathioprine for 24h on *E. coli* HM605 replication are shown. All fold replication is expressed relative to CFU seen at 6h in untreated macrophages. Ciprofloxacin (4 µg/ml) is the positive control. Kruskal Wallis non parametric ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N3, n9. Mean fold replication \pm SE is shown.

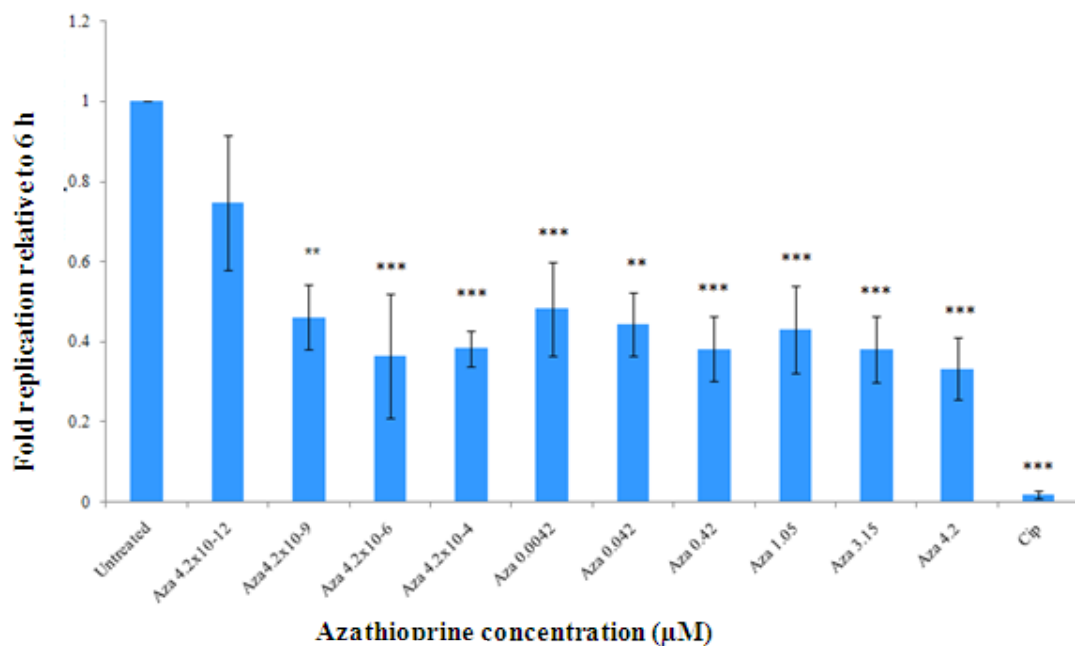


Figure 8.4 The effect of 24h pre-incubation of J774-A1 macrophages with clinically relevant concentrations of azathioprine on HM605 *E. coli* replication. Ciprofloxacin is the positive control. All doses examined (4.2×10^{-12} - $4.2 \mu\text{M}$) with the exception of the $4.2 \times 10^{-12} \mu\text{M}$ dose had a statistically significant inhibitory effect on *E. coli* replication. Kruskal Wallis non parametric ANOVA: ** $p < 0.01$, *** $p < 0.001$. N3-8, n9-24. Fold replication is expressed relative to CFU numbers at 6 h in untreated macrophages. Mean fold replication \pm SE is shown.

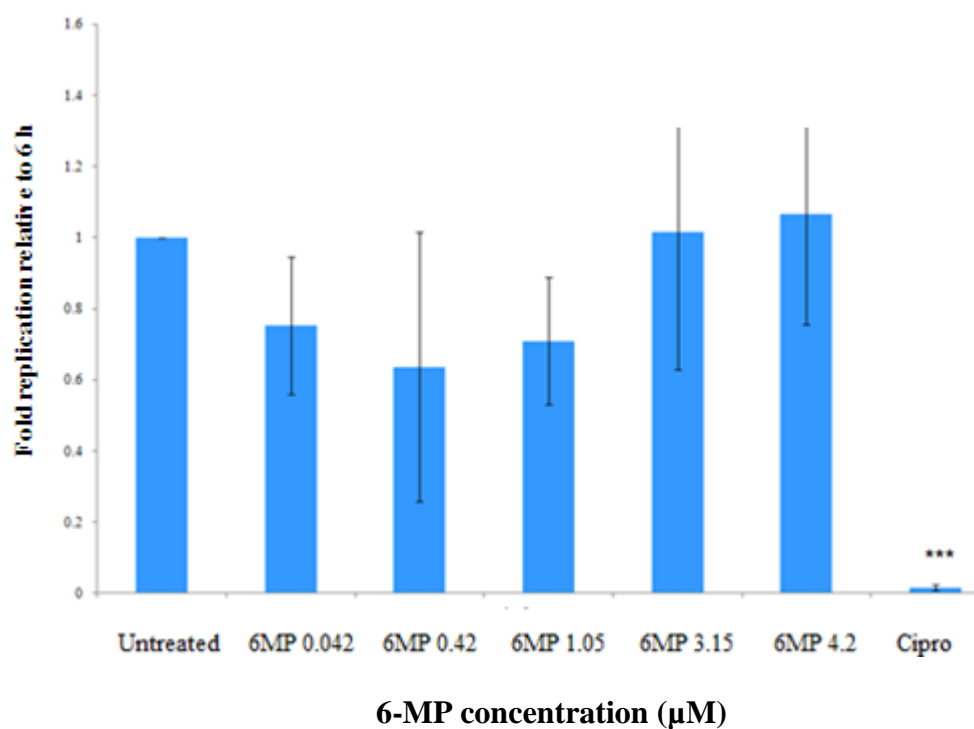


Figure 8.5 The effect of 24h pre-incubation of J774-A1 macrophages with 6-MP on intra-macrophage *E coli* replication. Kruskal Wallis non parametric ANOVA: *** $p < 0.001$. N3, n9. Fold replication is expressed relative to CFU numbers at 6 h in untreated macrophages. Mean fold replication \pm SE is shown.

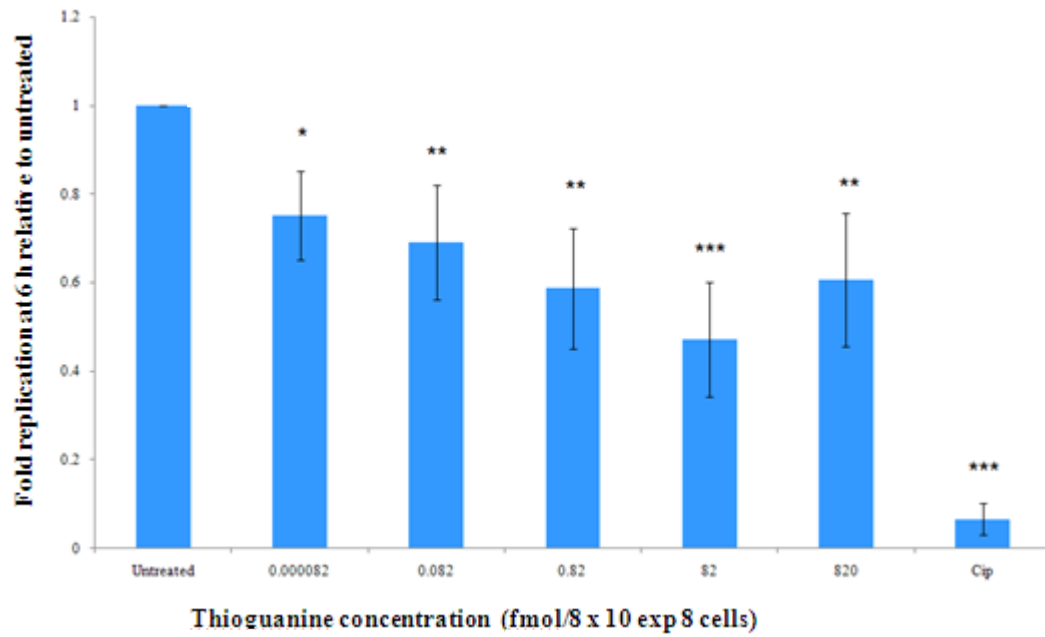


Figure 8.6 The effect of 24h pre-incubation with 6- TG on intra-macrophage *E. coli* HM605 replication Kruskal Wallis non parametric ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N3, n9. The 820 fmol/8x10⁸ cells dose is 10x the usual clinically therapeutic level. Fold replication is expressed relative to CFU numbers at 6 h in untreated macrophages. Mean fold replication \pm SE is shown.

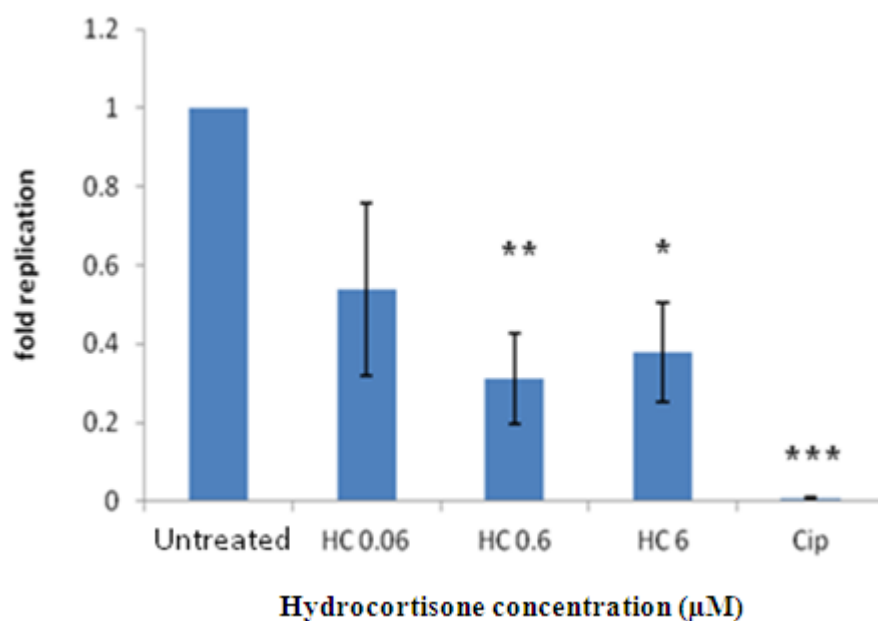


Figure 8.7 The effect of 24h pre-incubation with hydrocortisone on intra-macrophage *E. coli* HM605 replication. Kruskal Wallis non parametric ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N4, n12. Fold replication is expressed relative to CFU numbers at 6 h in untreated macrophages. Mean fold replication \pm SE is shown.

8.6.3 Assessment of the effect of therapeutic agents on macrophage growth and viability

To confirm absence of a direct toxic effect on macrophage cells of the immunomodulator drugs, two experiments were performed. Firstly an experiment was performed to assess cell growth, and secondly toxicity was assessed using an enzymatic assay.

Azathioprine and hydrocortisone were investigated for any potential toxic effects on macrophage cells that would alter interpretation of results. There were no effects seen with pre-incubation with these immunomodulator drugs on J774-A1

macrophage cell number and growth rate (Table 8.2). No significant differences were seen between treatment and control groups when compared on day 2 of the assay. Day 2 was when macrophages were infected after pre-incubation with the immunomodulators.

The possible cytotoxic effects of azathioprine and hydrocortisone as well as the *E. coli* HM605 effect on macrophage viability was also assessed using the Adenylate Kinase Cytotoxicity assay (Table 8.3). The kit uses luciferin to generate fluorescence when adenylate kinase is released from non viable cells. No significant differences were seen between cells treated with immunomodulator, *E. coli* and control groups. Signals produced by PBS, RPMI media, and the bacterial suspension alone are shown for comparison (Table 8.3).

Table 8.2 Experiment to evaluate the effect of peak concentrations (μM) of immunomodulator on J774-A1 macrophage cell growth. Kruskal Wallis non parametric ANOVA. N3, n12-18. AZA = azathioprine. HC = hydrocortisone. Cell numbers are expressed as average cell numbers per well \pm SEM. At day one cell counts were only assessed in untreated wells to confirm ongoing replication.

Drug exposure				
	Untreated	AZA 210 μM	AZA 4.2 μM	HC 6 μM
Day				
0	$1 \times 10^5 \pm 0$	$1 \times 10^5 \pm 0$	$1 \times 10^5 \pm 0$	$1 \times 10^5 \pm 0$
1	$1.82 \times 10^5 \pm 0.342$	N/A	N/A	N/A
2	$3.27 \times 10^5 \pm 0.856$	$2.26 \times 10^5 \pm 0.392$	$2.14 \times 10^5 \pm 0.406$	$2.56 \times 10^5 \pm 0.597$

Table 8.3 Adenylate Kinase (AK) Cytotoxicity Assay. AK signals in relative luminescence units (RLU) from PBS, *E.coli* and RPMI media are shown for comparison, expressed as mean \pm SE. AZA = azathioprine, HC = hydrocortisone. Kruskal Wallis non parametric ANOVA. N2, n6-27 (assessed on day 2 of experiments)

Cell exposure	Untreated	AZA 210 μ M	AZA 210 μ M + <i>E.coli</i>	AZA 4.2 μ M	AZA 4.2 μ M + <i>E.coli</i>	HC 6 μ M	HC 6 μ M + <i>E.coli</i>	<i>E.coli</i> alone	PBS	RPMI
AK level	23700 \pm	34400	38100	26600	30900	20100	34800	6530	2860	5370 \pm
Average	7510	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm	1560
RLU \pm		8690	5450	11200	2690	6660	9420	557	1490	
SEM										

8.6.4 Experiments to quantify any directly bacteriocidal effects of immunomodulator drugs on *E. coli* HM605

The effects of azathioprine, hydrocortisone, 6-MP and 6-TG when directly co-incubated with *E.coli* over a 6 h timecourse are shown in figures 8.8 and 8.9. Ciprofloxacin at 4 μ g/ml was used as a positive control. Hydrocortisone had no significant effect on *E.coli* survival compared with control at any timepoint. Interestingly azathioprine had a statistically significant killing effect on *E.coli* at 4 h and a similar effect at 5/6 h which, however, was not statistically significant (figure

8.9). 6-MP also demonstrated a statistically significant *E.coli* killing effect at 3,5, and 6 h (figure 8.8), which was interesting as its' modulation of macrophage phenotype had not produced a significant result (figure 8.5). 6-TG had an inhibitory effect on *E.coli* replication at 4-6 h which was statistically significant at 5 h (figure 8.8).

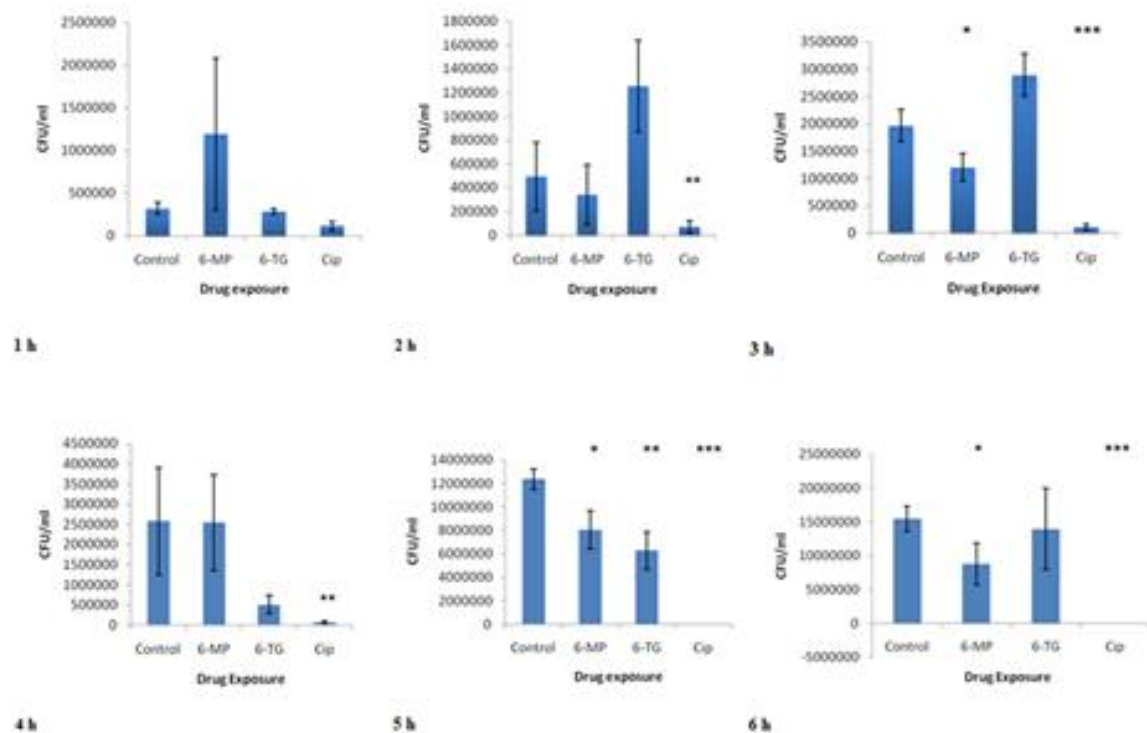


Figure 8.8 Exploration of a direct bacteriocidal effect of the immunomodulators 6-TG and 6-MP on *E.coli*. C = control, Cip = Ciprofloxacin. Ciprofloxacin concentration was C_{max} 4 μ g/mL, 6-MP at 4.2 μ M, and 6-TG at the equivalent concentration of 820 fmol per 8×10^8 cells. Kruskal Wallis non parametric ANOVA used to test significance relative to untreated bacteria. *p<0.05,**p<0.01, ***p<0.001. N2, n6. Absolute corrected CFU numbers are shown as mean \pm SE.

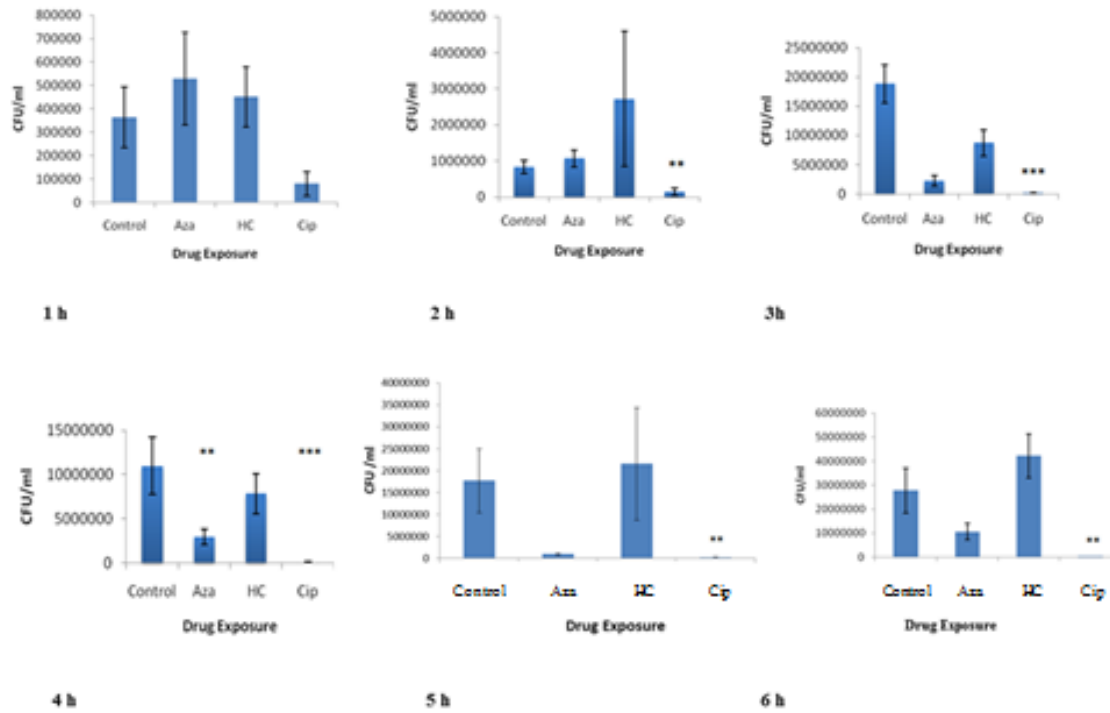


Figure 8.9 Exploration of a direct bacteriocidal effect of the immunomodulators Azathioprine and Hydrocortisone on *E.coli* growth. C = control, Cip = Ciprofloxacin. Ciprofloxacin concentration was C_{max} 4 μ g/mL, hydrocortisone concentration 6 μ M, and azathioprine 210 μ M. Kruskal Wallis non parametric ANOVA was used to test significance relative to untreated bacteria, *p<0.05, **p<0.01, ***p<0.001. N2, n6. Absolute corrected CFU numbers are shown as mean \pm SE.

8.7 Conclusions

Metronidazole did not demonstrate an inhibitory effect on intra-macrophage *E.coli* replication *in vitro* either as a sole agent, nor any additional effect when used in combination with ciprofloxacin, either at C_{\max} or 10% C_{\max} .

This work demonstrates a significant inhibition of intra-macrophage *E.coli* replication *in vitro* at clinically relevant concentrations of the immunosuppressives azathioprine and 6-TG, but not 6-MP.

Hydrocortisone unexpectedly inhibited intra-macrophage *E.coli* replication. The effects of hydrocortisone during sepsis *in vivo* may be mediated by other effects on the immune system.

The inhibitory effects of these drugs were not due to cytotoxic effects on macrophage cells.

At a clinically relevant concentration, 6-MP demonstrated a degree of direct bacteriocidal effect on *E.coli* despite absence of effect on intra-macrophage replication.

8.8 Discussion

Ciprofloxacin at C_{\max} and 10% C_{\max} concentrations significantly inhibited the replication of CD *E. coli* within murine macrophages, but metronidazole had no effect on *E. coli* killing within macrophages either when used alone or in combination with ciprofloxacin at C_{\max} and 10% C_{\max} . Azathioprine suppressed intra-macrophage *E. coli* replication at therapeutic concentrations ($> 2 \mu\text{M}$), but also at concentrations much lower than this, even as low as $4.2 \times 10^{-9} \mu\text{M}$. 6-MP did not significantly inhibit *E. coli* replication within macrophages at the clinically relevant concentrations used, but 6-TG did, with peak suppression at 6-TG concentration of 82 fmogles per 8×10^8 cells, the magnitude it is measured at in steady state for normal thiopurine dosing. The steroid hydrocortisone inhibited the replication of CD *E. coli* within macrophages at $0.6 \mu\text{M}$ but also at the clinically relevant concentration of $6 \mu\text{M}$. Experiments did not show any adverse effect of the drugs used on macrophage growth or viability with or without addition of *E. coli*. This confirmed that the inhibitory effects on bacterial replication seen with immunomodulators were due to true modulation of bacterial survival within macrophages. Hydrocortisone had no direct bacteriocidal effect on *E. coli* replication over a 6 h timecourse though the other immunomodulator drugs used, azathioprine, 6-MP and 6-TG did have a bactericidal effect at intermittent timepoints, with 6-MP having the earliest bacteriocidal effect after 3 h, in contrast to its negative effect on macrophage modulation. 6-MP was the only drug that demonstrated an inhibitory effect at a clinically relevant concentration, and produced 39 to 43% inhibition at 3, 5 and 6 h timepoints.

Ciprofloxacin was effective both at C_{\max} and 10% C_{\max} at inhibiting replication of *E. coli* within macrophages compared to bacterial replication in

untreated macrophages, this supports previous work from this laboratory and suggests the importance of this antibiotic's use in clinical trials. The use of metronidazole as an antibiotic to target the *E.coli* potentially relevant in CD pathogenesis is not supported by this *in vitro* work, as there is no evidence that it confers any additional benefit to intra-macrophage killing over ciprofloxacin used alone. Metronidazole might conceivably have had efficacy against *E. coli* in the microaerophilic environment within macrophage vesicles but this was not seen. However it has probable clinical efficacy in CD and this may be due to the effective killing of anaerobic bacteria which may infect tissue secondarily after mucosal ulceration has been initiated in response to other pathogenic agents. It also may have immunological effects, such as blockade of neutrophil recruitment which give it efficacy independent of its antibiotic effect (336). It may be valuable for treatment of anaerobic infection in abscesses and perianal disease (356). The use of metronidazole to control relapse in CD also has the support of meta-analysis (269), but can be limited by the unwillingness of patients to abstain from alcohol during a therapeutic course, or complications from protracted use such as peripheral neuropathy. Further clinical trials of antibiotic combinations in CD which target *E.coli* specifically are warranted. Constructing these trials and selecting antibiotic combinations needs careful consideration of issues including side effect profiles, microbial resistance, and interactions with drugs used in maintenance therapy in CD.

It was, and is, to some extent, difficult to determine what therapeutically relevant concentration of thiopurines are. There are many metabolic alternate cycling pathways and intermediaries (section 2.10.1) between azathioprine and its terminal metabolites which include thioguanine, and the level of activity in these enzymatic pathways can differ between patients (357), making modelling of thiopurine usage in

the *in vivo* system with *in vitro* techniques difficult. In this work data was utilised which demonstrated average thioguanine levels in thiopurine treated patients (352) and applied this to the macrophage cell numbers in our experiments which we had quantified, assuming these concentrations approximated to quantities found in erythrocytes. For azathioprine and 6-mercaptopurine, concentrations of clinical relevance had been defined in previous work (287;351).

The *in vitro* data examining the immunomodulators raise some interesting questions about the mode of action of some of the pharmacological agents used to treat IBD. The differing effects of azathioprine and its metabolites 6-MP and 6-TG are explicable. All these compounds generally had some bacteriocidal effect when directly co-incubated with bacteria, though generally tested at concentrations above those that were clinically relevant. The effect of 6-MP which was used at a concentration close to its therapeutic concentration was interesting as it had not modified intra-macrophage replication of *E.coli*. It may be that 6-MP is not taken up by cells in the *in vitro* model, and certainly azathioprine is intracellularly cleaved into its active metabolites following cell uptake. The presence of hypoxanthine in the RPMI culture media (358) may have effectively diluted the 6-MP before it could be utilised by the macrophage cells, as 6-MP is a hypoxanthine analogue. It may also be that high levels of xanthine oxidase, found in most murine tissues (359), reduced quantities of 6-MP in our *in vitro* model with their purine catabolising activity. Regarding the agents used in this work separately as an ‘antibiotic’ or as an ‘immunomodulator’ may also be an artificial simplification as they may possess properties of both, which has to some extent already been described. The mode of action of all the ‘immunomodulators’ to some extent seems to be related to altering sensitivity to reactive oxygen species (287;288), affecting the activity levels of iNOS

(292), and modulation of phagocytosis. This work prompts further examination of these mechanisms of action of azathioprine and its metabolites. Perhaps further *in vitro* murine work examining the effects of 6-MP on intra-macrophage *E.coli* replication would be improved co-incubation with allopurinol, to inhibit xanthine oxidase effects and careful selection or dialysis of FCS before use in culture media to avoid problems with hypoxanthine rich sera.

The suppression of intra-macrophage *E.coli* replication by hydrocortisone is interesting, as it is associated with an increased risk of sepsis *in vivo*. This work supports previous data suggesting steroids are pro-phagocytic (293), and their effects on sepsis *in vivo* may be more related to their suppression of neutrophil recruitment. It is probable that their effect on the body's response to sepsis may be mediated by blockade of the recruitment of neutrophils to a site of injury (360), which is mediated by IL-8 (202), or inhibition of macrophage recruitment (294), rather than by any direct effect on the ability of phagocytic leucocytes to kill bacteria. They were non bactericidal in direct co-incubation with bacteria, and this may be related to the timecourse they take to produce an effect rather than a genuine absence of effect. Alternatively it may be that steroids have differential effects on bacterial phagocytosis and killing at different timepoints.

It would be interesting to define the effects of other drugs on intra-macrophage *E.coli* replication, particularly those which have efficacy in CD such as anti-TNF α therapies, or other immunosuppressives such as ciclosporin or tacrolimus. It would also be informative to define the effects of the therapies used on the cell-bacterial cytokine signalling pathways involved.

Finally, it is intriguing that many of the pharmacological agents investigated here, demonstrate either a direct *E.coli* killing effect, or an indirect inhibition of the

intra-macrophage survival of bacteria. These drugs also have proven clinical efficacy in CD management, either in maintaining remission or treating relapse. This gives some indirect support to the hypothesis that *E.coli* growth and replication, and its regulation is fundamental in CD pathogenesis.

CHAPTER NINE : THE EFFECT OF SOLUBLE FIBRES ON ADHESION OF DIARRHOEAL PATHOGENS TO CACO2 CELLS

9.1 Hypothesis:

Soluble plant fibres (NSP), such as those from Plantain bananas, oats and apples may affect the adhesion of diarrhoeal pathogens to intestinal epithelial cells.

9.2 Aim:

To assess, using various *in vitro* modalities, whether dietary soluble fibres (Non-starch polysaccharide, NSP) block or promote the adherence of diarrhoea-causing bacteria and Crohn's-associated *E. coli* to intestinal epithelial cell lines.

9.3 Introduction

9.3.1 The effect of NSP on AIEC adherence to intestinal cell lines

Previous experiments by our group have demonstrated the inhibitory effect of soluble plantain fibre on the adhesion of CD-specific AIEC bacteria to intestinal cell lines (232), as well as an inhibitory effect on the translocation of AIEC and diarrhoeal

pathogens across M (microfold) cell epithelium both *in vitro*, and in *ex vivo* human Peyer's patches (69). The M cell is a specialised epithelial cell that accounts for about 5% of cells in the surface "dome" epithelium that overlies the lymphoid follicles of the colon, and the Peyer's patches in the small intestine which maintain immune homeostasis in the bowel (Introduction chapter 2). These initial findings prompted the current clinical trial of plantain fibre supplementation for maintenance of remission in CD (discussed in chapter 7). Plantain fibre was not the only foodstuff that demonstrated an inhibitory effect on bacterial adhesion *in vitro*, as broccoli NSP also demonstrated efficacy but apple and leek NSP did not show a significant effect (69). The effect of apple pectin on *E.coli* adherence to intestinal cell lines was uncertain as it had caused haemolysis in the haemagglutination assay used as a proxy for adherence and had not been further tested (232). Pectins are a specific form of NSP, being dietary and functional fibre composed of complex heteropolysaccharides and containing 1,4-linked α -D-galactosyluronic acid residues (361;362). They are also extractable from citrus fruits and have a known mild antibacterial effect against *E.coli*. In other work beta-D-glucans derived from oats have been shown to increase the Nuclear factor kappa B (NFkB) signalling in the small intestines of mice, but not the colon (363). As this signalling is generally pro-inflammatory, it was interesting to consider the possible effects of oat fibre on *E.coli* adhesion to intestinal cell lines.

9.3.2 Different Pathotypes of *E.coli*

There are a number of different pathotypes of *E coli* whose pathogenicity and method of epithelial interactions vary usually according to their virulence factors (See

introduction chapter 2, section 2.5 for a detailed account). Enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E.coli* (ETEC) interact with, colonise and invade gastrointestinal tissue in diverse ways to cause disease. Diarrhoeal illness is a common cause of illness and death worldwide, particularly in developing countries (244), and infantile diarrhoea cases are rising (245). In some series *E coli* was detected in 20% of cases where a bacterial cause was suspected (244;246) and diarrhoeogenic *E.coli* pathotypes (DEP) represent a high disease burden, particularly in areas like sub-Saharan Africa (244;247).

9.3.2.1 Types of EPEC

EPEC can be divided into ‘typical’ and ‘atypical’ types. They are distinguished from entero-haemorrhagic *E. coli* (EHEC) by absence of the phage borne shiga toxins (244). Typical EPEC strains carry genes for the LEE (locus for enterocyte effacement), virulence plasmids bearing genes encoding bfp (bundle-forming pili) and EAF (EPEC-adherence factor) whilst atypical EPEC do not have the EAF-plasmid or EAF-associated virulence plasmid (244;249). Their unique interaction with epithelia are described in section 2.5.1.

9.3.2.2 Types of ETEC

ETEC are amongst the most common causes of travellers' diarrhoea (244;254), and also a common cause of infantile diarrhoea, spread by contaminated food and water sources (255). Their pathogenesis is toxin mediated and is discussed in section 2.5.2. Although they promote diarrhoea by the effects of their toxins rather than by mucosal invasion, inhibition of bacterial proximity to the intestinal mucosa should reduce pathogenicity.

9.3.3 *C. Difficile* and *Campylobacter jejuni*

C. Difficile is the main cause of antibiotic-associated diarrhoea in hospitalised patients, commonly due to suppression of the normal gastrointestinal microbiota by broad spectrum antibiotics. It is a spore forming organism, and has the potential to cause pseudomembranous colitis which can be fatal (303;364). The diarrhoeal illness caused is toxin-mediated, utilising enterotoxin (*toxin A*) and cytotoxin (*toxin B*) (365), though approximation of the bacterium to the mucosa is probably mandatory for pathogenesis (366). *C. Difficile* PCR ribotype 027, (the ribotype investigated here) amongst others, is hypervirulent and is associated with increased mortality (367), probably through much higher levels of toxin production (368). This infection represents a significant financial burden to the NHS, with *C. Difficile* associated diarrhoea associated with costs of around £4107 per patient affected in the UK (369).

Campylobacter jejuni is a bacterium associated with enormous morbidity worldwide. It is a microaerophilic spiral shaped bacterium, that colonises birds, (chickens are important in the human food chain) and is associated with 14% of acute infectious cases of diarrhoea worldwide. It can produce watery diarrhoea, an appendicitis like syndrome, and neurological complications, namely the Guillain-Barré syndrome. Its methods of pathogenicity are under investigation as it lacks typical toxins or plasmid sited invasion factors, yet is able to invade and damage intestinal tissue (370).

The inhibitory effects of the NSP on CD AIEC bacterial adhesion have been examined, but the effect of NSP on other diarrhoeal pathogens has not previously been described. In the experiments described here the inhibitory effects of oat and plantain NSP as well as apple pectin on the adhesion of ETEC, EPEC, *C. difficile* and *Campylobacter jejuni* were evaluated as well as further assessment of AIEC inhibition by fluorescence-activated cell sorting (FACS). The utility of FACS for measuring bacterial to cell adherence had been demonstrated previously for *C.difficile* (303).

9.4 Methods

9.4.1 Cell culture

Caco2 cells were maintained as described in section 5.1.1.

9.4.2 Bacterial strains and growth conditions

EPEC, ETEC, *Campylobacter jejuni* and *C. difficile* strains were obtained and prepared as discussed in section 5.2.3, as was K12, (section 5.2.2) an indolent laboratory *E.coli* strain used as a negative control in the EPEC adhesion experiments. AIEC HM605 were prepared on LB agar as described in sections 5.2.1 – 5.2.2.

Prior to infection of cultured epithelial cells, bacteria were washed three times, re-suspended in sterile PBS and adjusted to an OD 550 nm or OD 570 nm equating to 1×10^9 CFU/mL. They were then diluted as necessary to produce an appropriate multiplicity of infection (MOI as per section 9.4.3).

9.4.3 Adherence assays

Bacterial strains were tested for their ability to adhere to, and/or invade, Caco2 cells in the presence of plantain NSP. Cells were maintained in complete DMEM at 37°C, 5% CO₂. Cells were initially seeded into 24-well tissue culture plates at 1×10^5 cells per well and grown overnight in DMEM (without antibiotics). The monolayers were then washed twice with sterile PBS. Bacteria were grown overnight on agar, washed twice in sterile PBS, and added to the well. Bacteria were added to each monolayer at an MOI of 10 - 500. For EPEC studies, bacteria were initially added at MOI of 10 but due to low adherence of EPEC to Caco2 cells, studies were repeated at higher MOI of 100 and 500. After 4 h of infection at 37°C, cell monolayers were washed 3 times with sterile PBS, and to enable quantification of total adherent and invasive bacteria they were lysed by adding 1% (v/v) Triton X-100 for 5 min to release

internalized bacteria. Ten-fold dilutions of the cell lysate were performed, and 50 µL from each was plated onto LB agar plates. Plates were incubated at 37°C, and CFU were enumerated after 24 h.

The effect of apple pectin and oat or plantain NSP on bacterial adhesion to Caco2 cells was assessed by pre-incubating Caco2 cells for 0.5 h with these NSPs before bacterial infection.

9.4.4 Preparation of soluble dietary fibre from plantain and oats, and apple pectin

Non-starch polysaccharide (NSP) from plantain, the banana family (*Musa* spp.) and oats were provided (Dr Niamh O’Kennedy, Provexis UK) as water soluble freeze dried powders. Concentrations tested were within the range of intraluminal concentrations that would be readily achievable with dietary supplementation (69). For the experiments evaluating the effect of plantain fibre on ETEC and EPEC bacterial adhesion, these concentrations were 0.005, 0.05, 5 and 50 mg/ml (ETEC experiments performed with Dr Carol Roberts). For the oat fibre and apple pectin experiments, a single 5 mg/ml concentration was used. 5 mg/ml was the dose which reflected likely luminal plantain concentrations present in the distal colon following a 5g twice daily patient supplementation with plantain, as per the clinical trial (chapter seven). The fibres and pectin were dissolved in PBS and sterile filtered before use. Apple pectin was used at a one in ten dilution of the stock concentration used in (232) i.e. at 5 mg/ml to facilitate dissolution.

9.4.5 Standardisation and analysis of results

The number of *E coli* added on the day of the infection was confirmed by performing ten-fold dilutions of the neat bacterial suspensions and plating 50µl of these dilutions on standard LB agar with the resulting number counted after overnight incubation at 37°C. These counts were used to produce a correction factor so bacterial numbers could be directly counted inter-experiment.

Data were analysed with total bacterial numbers (invasive and adherent) in Caco2 cells treated with NSP fibres or pectin compared to untreated cells. The datasets were statistically assessed using the Kruskal-Wallis non parametric ANOVA when multiple groups were involved. When two groups were directly compared, for example a comparison of bacterial strain and bacterial strain adherence following inhibitor pre-incubation with Caco2 cells, the analysis used depended on the variance present in the observations. Where variance was equal a paired t-test was used, and when data were not normally distributed a Mann-Whitney U test was performed to assess significance of the results. Differences were regarded as statistically significant when 2-tailed P values were < 0.05.

9.4.6 FACS adherence experiments

The potential inhibitory effect of plantain NSP on bacterial adhesion to Caco2 cells was evaluated for the bacteria AIEC (HM605), ETEC, *Clostridium difficile*, and *Campylobacter jejuni* using FACS. The method was based on (303). The sources of the bacteria, and their growth conditions are described in sections 5.2.1 – 5.2.3.

9.4.6.1 Cell culture for FACS

Caco2 cells were maintained as described in section 5.1.1 until 80% confluent in flasks. 24 h before adherence assays, DMEM medium was removed, cells were washed three times in cold PBS, and cells were incubated with antibiotic-free medium. Cells were then washed three times again with PBS and removed from tissue-culture flasks with a trypsin solution (as described in section 5.1.1). Cells were counted using a haemocytometer and adjusted to 1×10^5 cells/ml using antibiotic free DMEM.

9.4.6.2 Preparation of NSP for FACS

During pilot experiments it was noticed that NSP at concentrations of 20 mg/ml on its own produced a FACS signal (results not shown). This was not seen when NSP was prepared at 5 mg/ml.

Plantain derived NSP (NSP#3G, batch 17+18 containing 55.7 % plantain fibre and plantain-derived maltodextrin at 33.4 %) was dissolved in sterile PBS to give a concentration of 20 mg/ml (for a final experimental concentration of 10 mg/ml).

Other plantain concentrations were prepared by appropriate dilution of this initial solution with sterile PBS, i.e. 10, 5 and 2 mg/ml, for final experiment concentrations of 5, 2.5, and 1 mg/ml.

9.4.6.3 Bacterial preparation for FACS experiments

C. difficile was cultured by placing one Protect bead into 50 ml Fastidious Anaerobe Broth and culturing anaerobically without shaking for 36 h at 37°C.

ETEC also had been stored on Protect beads at -80°C and were cultured by placing one bead into 40 ml LB broth and culturing aerobically without shaking for 24 h at 37°C.

AIEC HM605 were cultured by placing a loop-full of inco-loop from Protect beads into 40 ml LB broth and culturing aerobically without shaking for 24 h at 37°C.

9.4.7 Harvesting bacteria and fluorochrome labelling

Bacteria were harvested by centrifugation at 9000 rpm (19017g) for 5 min and resuspension in sterile PBS. Bacteria in suspension were then quantified by OD reading at 550 nm, and were adjusted to 1×10^8 bacteria/ml (OD=0.825, then diluted ten fold for fluorochrome labelling.

1 mM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF/AM) stock solution (supplied by Invitrogen), had been divided into 50 µl aliquots which were stored in a well-sealed container and protected from the light with aluminium foil and stored in the -20°C freezer. For a 1 µM working concentration 10 µl stock solution of BCECF/AM was added for every 10 ml bacterial suspension (1×10^8 bugs/ml).

Bacteria were labelled with BCECF/AM fluorochrome at 37°C for 60 min ensuring that tubes were protected from the light with aluminium foil prior to incubation. Excess fluorochrome was removed by 5 sequential washes in PBS followed by centrifugation at 9000 rpm (19000 g) for 5 min.

9.4.8 NSP plantain treatment of the bacterial suspensions

After the penultimate wash, each bacterial suspension was split into an appropriate number of aliquots (for the plantain concentrations assessed on the day of the experiment, 2-5) before centrifugation. After the final wash bacteria were re-suspended in PBS containing 20,10,5, or 2mg/ml plantain NSP, or PBS alone and were incubated for 30 min ensuring tubes were protected from the light using aluminium foil.

9.4.9 FACS Adherence assay

An equivalent volume of Caco2 cells at 1×10^5 cells/ml was added to the fluorescently labelled bacteria and incubated at 37°C for 1 h. (i.e MOI 1000) After incubation, cells were washed by centrifugation at 1200 rpm (340 g) for 5 mins x 3 in PBS to remove any non-adherent bacteria. After the final wash the pellets were resuspended in 0.5 ml PBS and were transferred into individual 1 ml tubes.

Caco2 cells with and without adherent *C. Difficile*, *ETEC*, *Escherichia coli* or *Campylobacter jejuni* in the presence or absence of varying concentrations of plantain fibre were measured in a FACScan flow cytometer. A total of 10,000 events were

acquired per cell and bacterial suspension, and the data were analysed with the Cell Quest software programme. Results were expressed as Mean Fluorescent intensity (MFI) using the geometric mean of each sample to assess bacterial adherence to Caco2 cells. Student's t-test was used to compare differences in bacterial adherence in the presence or absence of soluble plantain fibre, for *C. difficile* and ETEC FACS studies, but when multiple NSP concentrations were used, a multiple group comparison with a non parametric ANOVA was used, (Kruskal Wallis), with significance noted for p values < 0.05.

Fluorescence of HM605 *E coli*, *C.difficile*, *ETEC* and *Campylobacter* alone was also measured but could not be compared directly to the other experimental groups due to different FACS gating requirements to measure bacteria alone. These samples were kept in case of any problems with getting a positive fluorescence signal for cells and bacteria together, but this problem did not materialise.

9.5 Results

9.5.1 The effect of plantain NSP, oat NSP, and apple pectin on ETEC adhesion to Caco2 cells

Pre-incubating the Caco2 cells with a 5 and 50 mg/ml concentration of plantain NSP significantly inhibited the adherence of ETEC bacteria to the cells (figure 9.1).

Pre-incubation of Caco2 cells with a 5 mg/ml concentration of apple pectin did not significantly inhibit the adhesion of ETEC to the Caco2 cells (figure 9.2). Oat NSP fibre also had a non-significant inhibitory effect when it was used to pre-

incubate Caco2 cells at a concentration of 5 mg/ml prior to infection of the cells with the ETEC bacteria (figure 9.3).

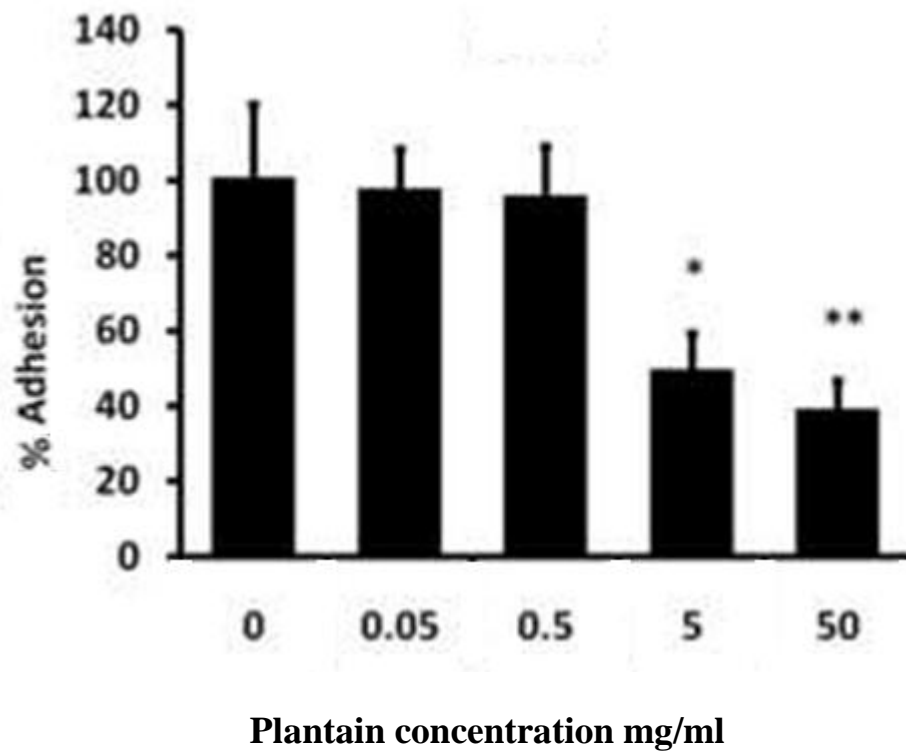


Figure 9.1 The effect of plantain NSP on ETEC adhesion to Caco2 cells at differing doses. ETEC adhesion to Caco2 cells was significantly inhibited at concentrations of 5 and 50 mg/ml plantain NSP. (N3,n9 Kruskal Wallis non parametric ANOVA * = $p < 0.05$ ** = $p < 0.01$). Mean % adhesion \pm SE is shown.

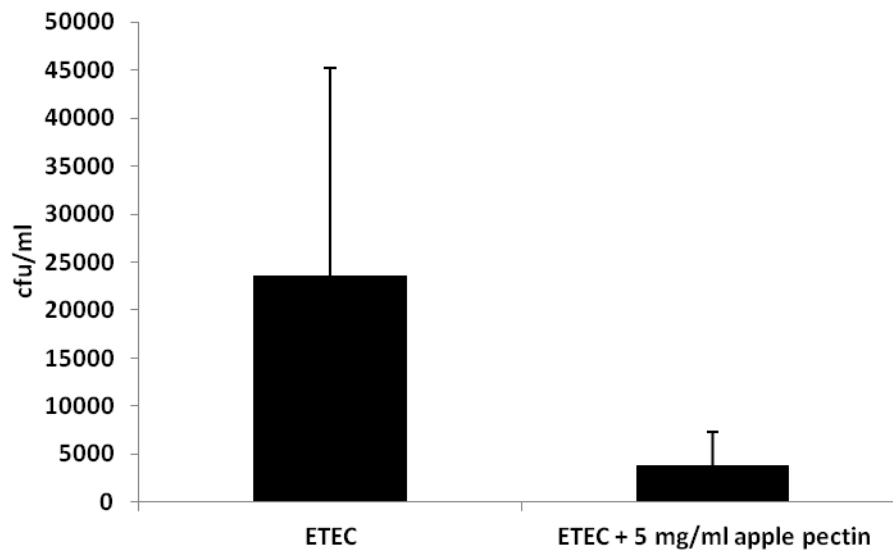


Figure 9.2 Apple pectin non significantly inhibited the adherence of ETEC to Caco2 cells. Apple pectin used at a concentration of 5 mg/ml non significantly inhibited ($p = 0.22$) the adhesion of ETEC to Caco2 cells (N2, n6 Mann-Whitney U test). Mean cfu/ml \pm SE are shown.

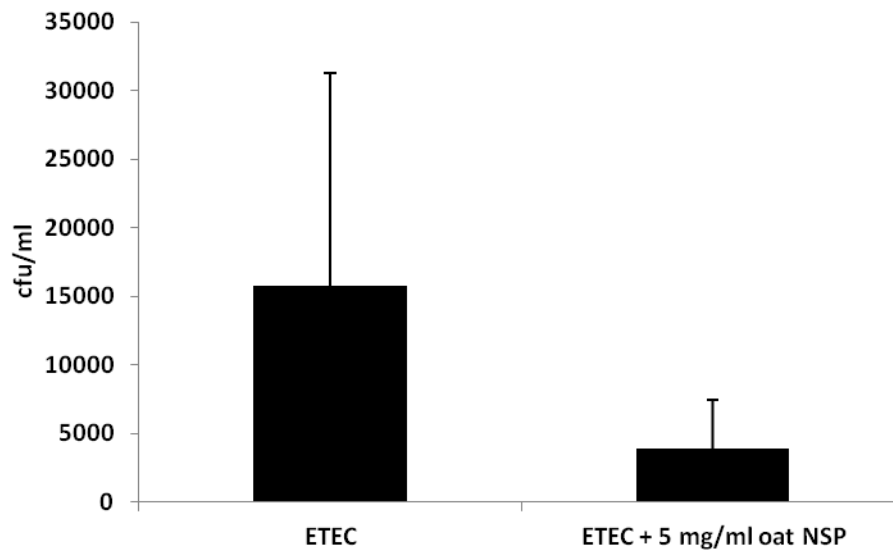


Figure 9.3 Oat NSP non significantly inhibited the adherence of ETEC to Caco2 cells. Oat NSP used at a dose of 5 mg/ml non significantly inhibited ($p=0.30$) the adhesion of ETEC to Caco2 cells (N3, n9 Mann-Whitney U test). Mean cfu/ml \pm SE are shown.

9.5.2 The effect of plantain NSP on EPEC adhesion to Caco2 cells

Plantain NSP had no effect on the binding of the typical D55 (figure 9.4), or atypical e2348/69 (9.5), EPEC strains to Caco2 cells *in vitro* at any dose investigated (0 - 50 mg/ml). The AIEC HM605 has significantly greater binding to Caco2 cells than both the EPEC bacteria and the negative control bacterium K12. Increasing the EPEC MOI to 500:1 did not affect this result (data not shown). Pre-incubating the Caco2 cells with a 5 mg/ml concentration of plantain NSP significantly inhibited the binding of the AIEC HM605 to Caco2 cells, as had been seen previously, demonstrating that both the positive and negative controls worked. Pre-incubating the Caco2 cells with a 5 mg/ml concentration of plantain NSP did not significantly inhibit the adherence of

the K12 bacteria to the Caco2 cells. In the absence of inhibitor AIEC CD *E. coli* HM605 bacteria were significantly more adherent to Caco2 cells than both the K12 and EPEC bacteria.

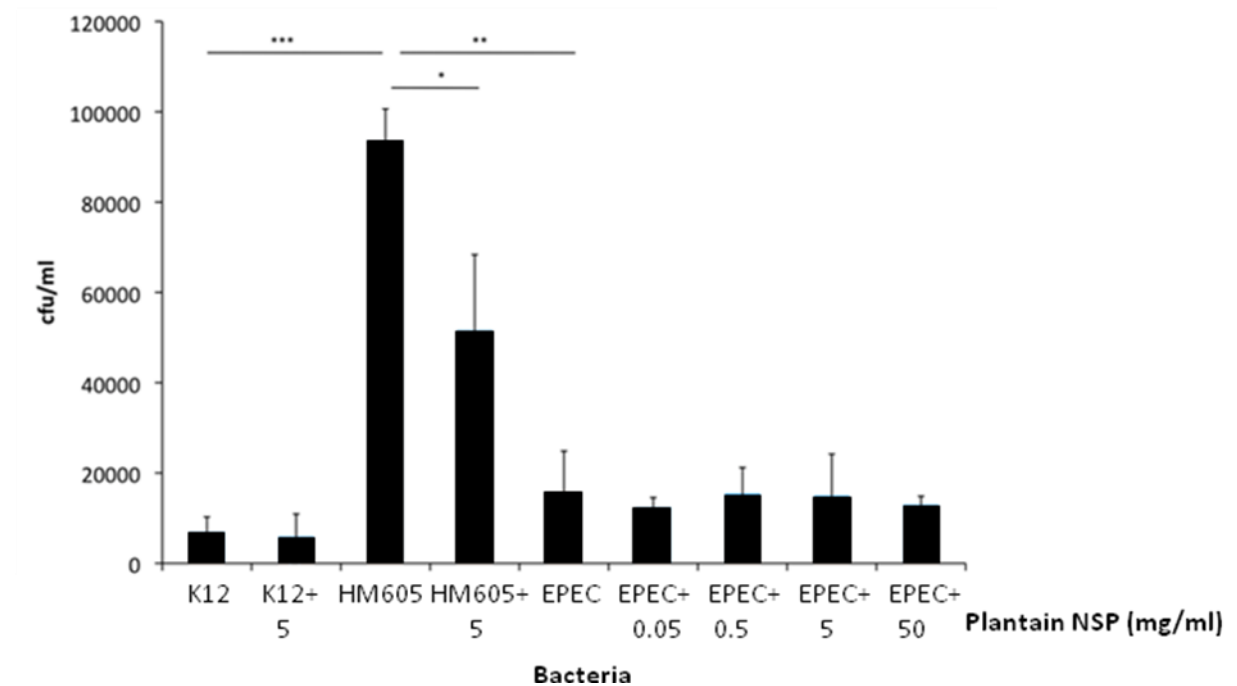


Figure 9.4 Plantain NSP has no effect on the binding of the EPEC strain D55 to Caco2 cells. Histogram bars are labelled with the name of the bacterial strain + concentration in mg/ml of NSP used in preincubation of cells if present. No dose of plantain in the range tested (0.05-50 mg/ml) had any effect on the binding of EPEC strain D55 to the Caco2 epithelial cells (N3, n9 Kruskal Wallis non parametric ANOVA * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$). Mean cfu/ml \pm SE are shown.

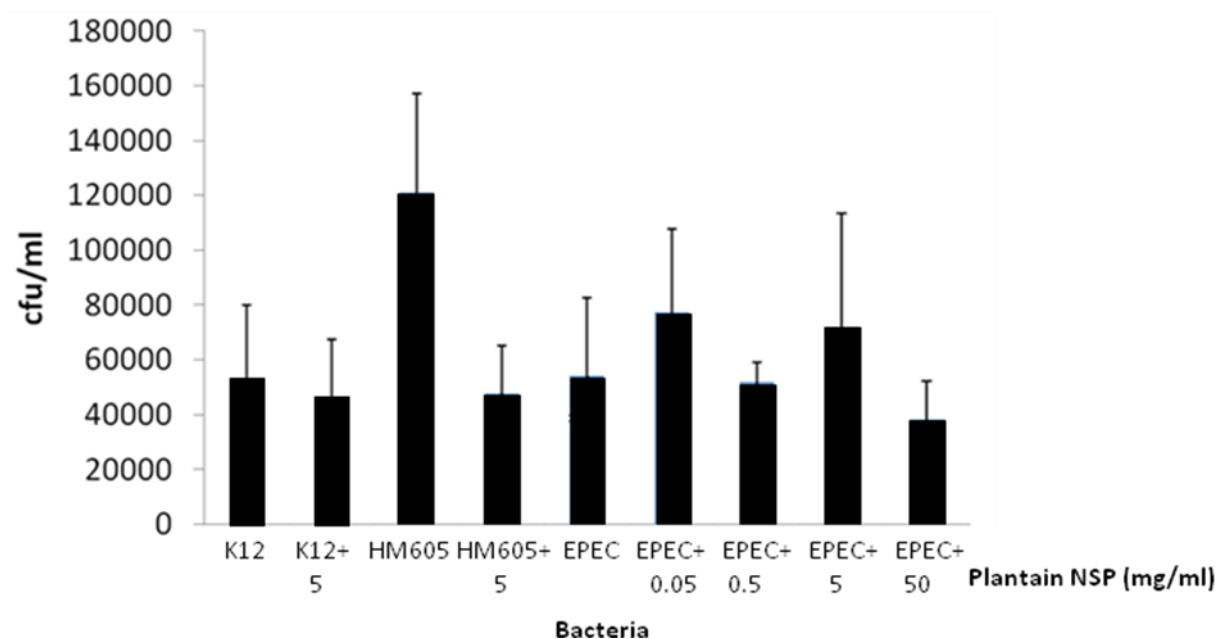


Figure 9.5 Plantain NSP has no effect on the binding of the atypical EPEC strain e2348/69 to Caco2 cells. Histogram bars are labelled with the name of the bacterial strain + concentration in mg/ml of NSP used in preincubation of cells. No dose of plantain in the range used (0.05-50 mg/ml) had any effect on the binding of the atypical EPEC strain to the Caco2 epithelial cells. (N3, n9 Kruskal Wallis non parametric ANOVA). Mean cfu/ml \pm SE are shown.

9.5.3 FACS assessment of bacterial adherence and its inhibition by soluble fibres

9.5.3.1 The effect of NSP plantain fibre on AIEC adherence to Caco2 cells

When AIEC bacteria were pre-incubated with plantain NSP concentrations between 2.5 and 10 mg/ml, inhibition of AIEC adherence to Caco2 cells was observed at all concentrations with a peak inhibition seen at 2.5 mg/ml (with P value 0.054 just

failing to reach conventional significance, (figure 9.6). The reduced inhibition of AIEC bacteria at higher NSP concentrations was not typical for an inhibitor, and was not consistent with previously published results that demonstrated a dose response effect (69). This was perhaps explained by the FACS signals observed from plantain fibre used alone at higher concentrations (9.4.6.2). The results seen with inhibition at 5 mg/ml are in agreement with the tissue culture results in section 9.5.2 (figures 9.4 and 9.5).

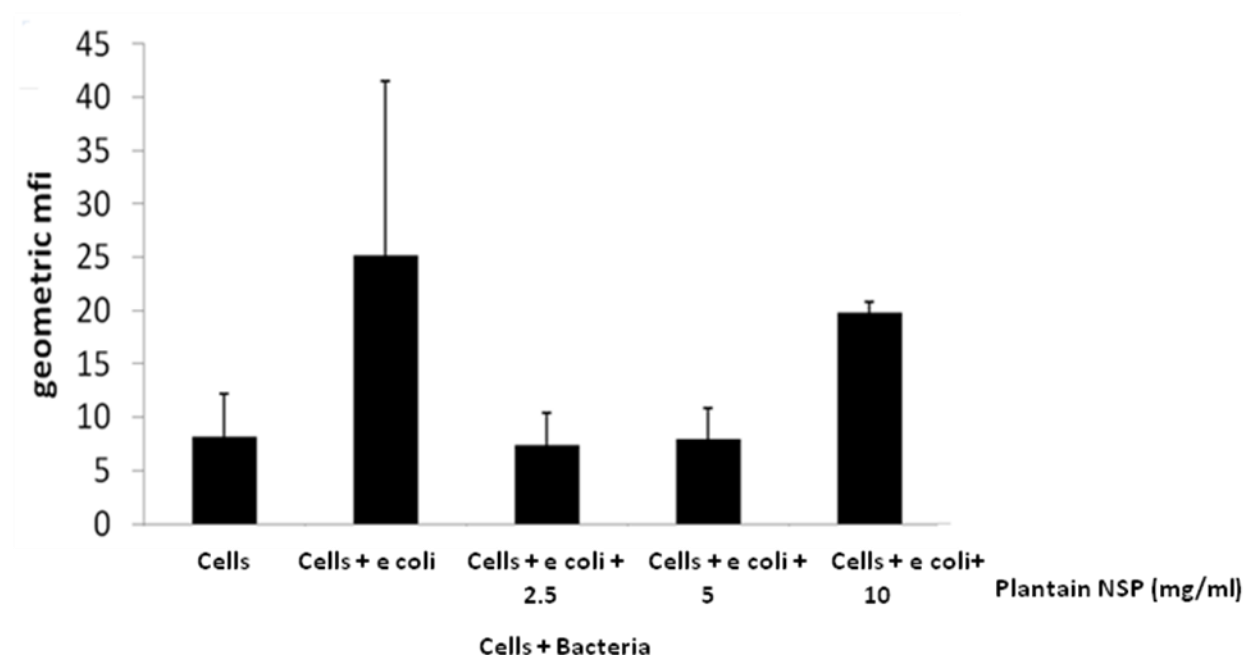


Figure 9.6 The effect of plantain NSP at different doses on AIEC adhesion to Caco2 cells. Concentration of plantain NSP used in pre-incubation of AIEC before addition to Caco2 cells is noted below each histogram bar. Adherence is expressed as geometric mean fluorescence intensity \pm S.E. The signal from Caco2 cells alone, cells with *E. coli* added and the effect of pre-incubating the bacteria for 30 min before addition to the cells with doses from 2.5 – 10 mg NSP plantain is shown. There is an inhibition of *E. coli* binding to Caco2 cells when plantain NSP is used at a dose of 2.5 mg/ml ($p=0.054$), which is seen at higher doses to a lesser extent (N2-4, 10000 events per experiment, Kruskal Wallis non parametric ANOVA).

9.5.3.2 The effect of plantain NSP on *Campylobacter jejuni* adhesion to Caco2 cells

When *Campylobacter jejuni* bacteria were pre-incubated with plantain NSP concentrations between 1 and 10 mg/ml, non significant inhibition of *Campylobacter jejuni* adherence to Caco2 cells was observed at all concentrations. The higher FACS signals seen at higher plantain NSP concentrations in figure 9.6 were not seen in the FACS experiments with *Campylobacter*.

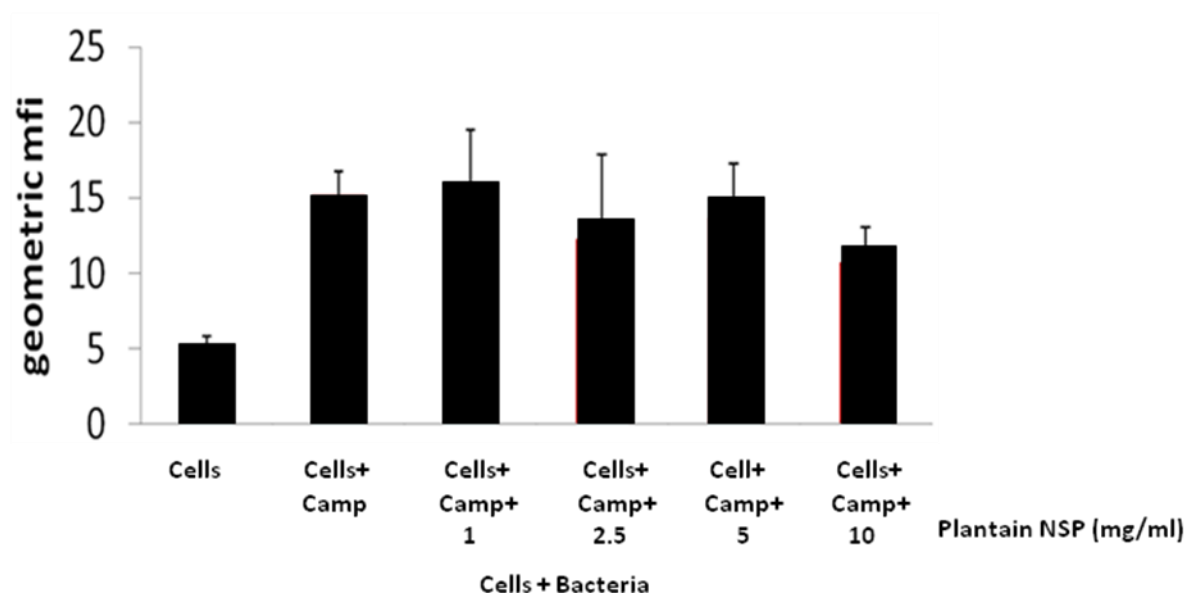


Figure 9.7 The effect of plantain NSP at different doses on *Campylobacter jejuni* adherence to Caco2 cells. Camp = campylobacter. Concentration of plantain NSP used in pre-incubation of *Campylobacter jejuni* before addition to Caco2 cells is noted below each histogram bar. Adherence is expressed as geometric mean fluorescence intensity \pm S.E. The signal from Caco2 cells alone, cells with *Campylobacter jejuni* added and the effect of pre-incubating the bacteria for 30 min before addition to the cells with doses from 1 - 10mg NSP plantain is shown. (N2-4, 10000 events, Kruskal Wallis non parametric ANOVA).

9.5.3.3 The effect of plantain NSP on *Clostridium difficile* and ETEC adhesion to Caco2 cells

The effect of pre-incubation with plantain NSP on *Clostridium difficile* and ETEC adhesion to Caco2 cells is shown in figure 9.8. There was a statistically significant inhibition of *C. difficile* and ETEC binding to Caco2 cells following pre-incubation with plantain NSP at 10 mg/ml ($p = 0.01$ and $p = 0.016$ respectively).

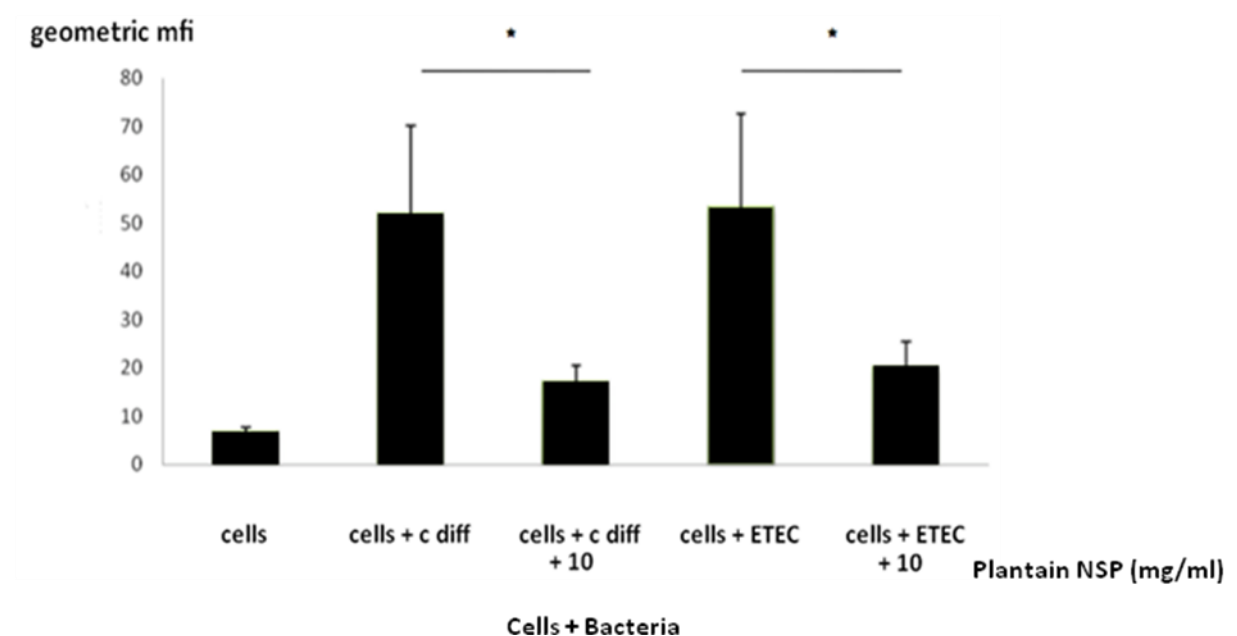


Figure 9.8 The effect of plantain NSP on *C.difficile* and ETEC adhesion to Caco2 cells. The bacterial species is noted below each histogram bar. In these experiments a plantain NSP concentration of 10 mg/ml was used to pre-incubate the bacterial species before addition to the Caco2 cells. Adherence is expressed as geometric mean fluorescence intensity \pm S.E. The signal from Caco2 cells alone, cells with. *C. difficile* or ETEC added, and the signal produced when the bacteria had been pre-incubated for 30 min with a concentration of plantain NSP of 10 mg/ml are shown (N4, 10000 events per experiment, students t test * = $p<0.05$).

9.5.3.4 FACS signals seen from NSP plantain fibre in isolation

In addition, (data not shown), FACS signals from NSP plantain fibre in isolation were seen when a concentration of 20 mg/ml were used, but not with a concentration of 5 mg/ml. These were the only concentrations evaluated, but this effect may conceal significant inhibitory effects of plantain at concentrations greater than 5 mg/ml, and clearly represents a significant drawback of FACS as a technique for estimating inhibition of adherence by soluble fibre.

9.6 Conclusions

NSP plantain has a statistically significant dose dependent inhibitory effect on the adhesion of ETEC to Caco2 intestinal cells, and inhibits ETEC adherence at concentrations that would be readily achievable in the colonic lumen (5 mg/ml) with dietary supplementation. This inhibitory effect was seen with two types of *in vitro* assay, both culture plate assays and FACS.

Oat NSP and apple pectin, both used at concentrations of 5 mg/ml, inhibited the binding of ETEC bacteria to Caco2 cells, but not with statistical significance. Further experiments to increase replication numbers are indicated but were limited during this work due to time constraints.

Plantain NSP had no effect on the binding of EPEC bacteria to Caco2 cells at any concentration between 0.05 and 50 mg/ml, though these experiments confirmed earlier work demonstrating a statistically significant inhibitory effect of plantain on CD AIEC bacteria adherence at concentrations of 5 mg/ml.

The FACS experiments demonstrated a statistically significant inhibition of ETEC bacteria with NSP plantain at 10 mg/ml, which supported the cell culture results, and also a significant inhibitory effect of plantain fibre on *C.difficile* adherence to Caco2 cells, also at 10 mg/ml.

FACS methods did not demonstrate a significant inhibition by plantain NSP of AIEC adhesion to Caco2 cells at any dose, though a 2.5 mg/ml concentration was almost

significant. No inhibitory effect of plantain on *Campylobacter jejuni* adherence to Caco2 cells was seen either though doses above 10 mg/ml were not used in either of these experimental series.

The significant FACS fluorescent signal generated by NSP plantain fibre in isolation has to be considered a significant drawback with the FACS assay. Conclusions based on the FACS assay alone have to be made with caution. It is not a suitable assay to use in further experiments to assess inhibition by soluble fibres.

9.7 Discussion

These experiments confirm that plantain NSP fibre has inhibitory effects on adhesion of AIEC and also some diarrhoeal pathogens to an intestinal cell line *in vitro*. The plantain fibre inhibited the adhesion of ETEC and *C. difficile* to Caco2 cells. It would be advantageous to perform further experiments with apple pectin and oat NSP fibre as there may be a real difference in the effects of these foods on ETEC bacterial adhesion to intestinal cells that can only be demonstrated with further replication. Dose response curves would also provide additional information. These experiments were restricted here due to time constraints.

The absence of an inhibitory effect of NSP plantain fibre on the adherence of both typical and atypical EPEC bacteria to Caco2 cells has some biological plausibility, as the EPEC bacteria use a unique mechanism of interaction with host epithelial cells. By secretion of its own translocation intimin receptor onto the host cell, which functions as a receptor for its intimin ligand (253), the EPEC may bypass the ‘mucosal blockade’, or cross linking of its’ own bacterial cell surface receptors, which will include lectin carbohydrate moieties. Soluble plantain fibre is a complex mixture of oligosaccharides, and any of these, perhaps particularly mannose, could interfere with the EPEC adhesion to the mucosal surface if the bacterium didn’t utilise these alternate host interaction strategies. Perhaps the atypical EPEC uses the proteins expressed from its mobility and resistance plasmids to avoid plantain cross linkage. The fact that both the negative and positive control experiments in the EPEC experiments gave the anticipated results supports the conclusion that this is a true result.

It was of interest that the oat fibre did not stimulate bacterial adhesion to Caco2 cells as might be hypothesised from its stimulatory effects on NF κ B in mice (363). This may result from different modulation of NF κ B signalling in mice and humans. Also of note, the ETEC and *C.difficile* adherence to Caco2 cells was inhibited by plantain NSP, and this could be clinically very useful, partly because there are suggestions that toxin production is not the sole mechanism for disease activity, but also because mucosal proximity is a factor in toxin-mediated damage.

The observation that NSP plantain fibre has its own FACS fluorescence signal when higher concentrations are used makes it difficult to use this technique in quantitative assays, and is a significant drawback. However, the plantain fibre could be run on its own as a control in each FACS adhesion experiment. The signal seen with higher plantain concentrations did not seem to distort FACS results, except perhaps with AIEC. Overall it does not seem appropriate to use the technique in further plantain adhesion assays, but the ability to measure 10000 cell-bacterial events in an experiment was a powerful technique and dual labelling both cells and bacteria in FACS adhesion experiments with fluorochrome is a possible alternative for future assays.

The potential for translating the results of these *in vitro* experiments into clinical trials investigating the prophylaxis of diarrhoeal pathogen infection, to complement the trials already underway investigating the use of plantain fibre in CD is clear (see chapter seven). When a 5 g twice daily supplement of NSP plantain fibre is given to a patient, the resultant minimum intra-luminal concentration of the foodstuff is estimated to be at least 5 mg/ml, even in the distal colon (232), and the work in this chapter has demonstrated *in vitro* efficacy at those concentrations of soluble fibre.

Diarrhoeal illness is a large burden worldwide, in terms of morbidity, mortality and cost, and antibiotics are often implicated as a cause of diarrhoeal illness. Additional strategies for prophylaxis of infective diarrhoea would augment current infectious disease prevention strategies. If dietary supplements are shown to be effective, it would result in lower incidence of multi-resistant bacteria and be very cost-effective. It could be of particular interest to focus on at risk groups, for example hospitalised patients over the age of sixty five who are on antibiotics, African infants and children in areas of poor sanitation, or people travelling frequently to endemic areas. Many of these diarrhoeal pathogens can also trigger a relapse of UC or CD (47;224) so the benefit would not solely be confined to those patients at risk of diarrhoeal illness, but could also prevent CD relapses.

CHAPTER TEN : SUMMARY OF PRINCIPAL FINDINGS

The development of qPCR assays for the quantification of *E. coli* bacteria and human tissue

There was a lengthy period of development, which involved assessment of a number of different techniques, for the development of standardised assays suitable for quantification of *uidA* genes and therefore by proxy *E.coli* in CD biopsies.

- **The optimal assay for producing standards for quantification of *uidA* genes proved to be the multi-step process of producing a suitable amplicon, splicing it into DNA, transforming cells to accept plasmid DNA and ultimately extracting the plasmid DNA from these clones and confirming the presence of the amplicon by qPCR with or without restriction enzyme digest.**

Bacterial numbers could be quantified with great precision with this assay with appropriate sensitivity (1140 copies), efficiency (104%), error (0.130), coefficient of variation or reproducibility (3.72 %), and correlation coefficient (0.995). The linearisation of the standard circular plasmid DNA template proved critical for the *uidA* assay, and this assay fulfilled the specified statistical pre-requisites for quantification of *E.coli* in the biopsies from CD tissue.

The removal of bacterial CFU quantification from the standardisation process improved assay performance substantially, as did accurate genomic and plasmid DNA quantification.

The use of the 18S rRNA kit to produce standardisation curves for quantification of human tissue produced an assay with a sensitivity of 5 human genomes, efficiency 104%, error 0.185, correlation coefficient 0.992, and coefficient of variation or reproducibility of 2.84 %, which fulfilled requirements for the denominator assay for biopsies from CD tissue. Using biopsy mass as a denominator did not produce differences in graph shape, supporting the contention that the 18S rRNA genome denominator was unaffected by presence or absence of human inflammatory as well as total cells.

Comprehensive experimental details were provided so that all these PCR assays could be repeated by other laboratories, along with the detailed statistical information above in line with published (MIQE) guidelines for PCR work.

Use of the qPCR assays for quantification of *E. coli* bacteria in CD biopsies

- **The assay works well to quantify the bacteria in biopsies and mucus washings from clinical trial samples of large intestinal tissue.**

There was a significantly lower quantity of *E.coli* bacteria in the combined mucus washings and biopsies from patients who were in clinical remission at three months compared to the same individuals (also in remission) at the start of the trial period. Although the data are blinded this implies a reduction in mucosa-associated bacteria with the trial intervention (plantain fibre or maltodextrin placebo).

It was not possible to demonstrate a statistically significant difference in *E. coli* bacteria present in the biopsies of (n=3) patients who relapsed clinically, compared to the bacteria present in the same patient during their period of remission but further studies are ongoing.

The effects of antibiotics and immunomodulators on CD *E.coli* replication within macrophages

- **Ciprofloxacin at C_{\max} and 10% C_{\max} concentrations significantly inhibited the replication of CD *E. coli* within murine macrophages, but metronidazole had no effect on *E.coli* killing within macrophages either when used alone or in combination with ciprofloxacin at C_{\max} and 10% C_{\max} .**
- **Azathioprine suppressed intra-macrophage *E.coli* replication at therapeutic concentrations. 6-MP did not significantly inhibit *E. coli* replication within macrophages at the clinically relevant concentrations used, but 6-TG inhibited bacterial replication within macrophages at therapeutically relevant levels**
- **Hydrocortisone inhibited the replication of CD *E.coli* within macrophages at clinically relevant concentrations.**

The macrophage cell viability experiment and adenylate kinase toxicity assay did not demonstrate any direct cellular toxicity from incubating macrophage cells with peak concentrations of azathioprine and hydrocortisone even after 24 h. This was not affected by the addition of *E.coli* to the cells, confirming that the inhibitory effects on bacterial replication seen with immunomodulators were due to modulation of bacterial survival within macrophages, rather than macrophage cell death.

Hydrocortisone had no direct bacteriocidal effect on *E.coli* replication over a 6 h timecourse though the other immunomodulator drugs used, azathioprine, 6-MP and 6-TG did have a bactericidal effect at intermittent timepoints. 6-MP was the only drug assessed near its usual therapeutic concentration and had the earliest bacteriocidal effect after 3 h, in contrast to the negative effect seen with the drug on macrophage modulation.

The effects of soluble fibres on AIEC and diarrhoeal pathogen adhesion to intestinal cells

- **NSP plantain has a dose dependent inhibitory effect on the adhesion of ETEC to Caco2 ileocaecal intestinal cells, and inhibits ETEC adherence at concentrations that would be readily achievable in the colonic lumen (5 mg/ml) with dietary supplementation. This inhibitory effect was seen with two types of *in vitro* assay, both culture plate assays and FACS.**
- Oat NSP and apple pectin, both used at concentrations of 5 mg/ml, inhibited the binding of ETEC bacteria to Caco2 cells, but not with statistical significance and further studies are needed to assess this further.
- Plantain NSP had no effect on the binding of EPEC bacteria to Caco2 cells at any concentration between 0.05 and 50 mg/ml, though these experiments confirmed earlier work demonstrating a statistically significant inhibitory

effect of plantain on CD AIEC bacteria adherence at concentrations of 5 mg/ml

- The FACS experiments demonstrated a statistically significant inhibition of ETEC bacteria with NSP plantain at 10 mg/ml, which supported the cell culture results, and also a significant inhibitory effect of plantain fibre on *C.difficile* adherence to Caco2 cells, also at 10 mg/ml.
- FACS methods did not demonstrate a significant inhibition by plantain NSP of AIEC adhesion to Caco2 cells at any dose, though a 2.5 mg/ml concentration was almost significant. No inhibitory effect of plantain on *Campylobacter jejuni* adherence to Caco2 cells was seen either though doses above 10 mg/ml were not used in either of these experimental series.

A fluorescent effect of the plantain fibre itself made interpretation of the FACS adherence experiments problematic, and suggests that it is an unsuitable assay to use in further experiments assessing the inhibition by soluble fibres of bacterial adhesion to intestinal cells.

CHAPTER ELEVEN : DISCUSSION AND IMPLICATIONS FOR FUTURE STUDIES

The qPCR assays of linearised plasmid DNA (chapter 6) were developed to examine the quantities of *E. coli* in colonic tissue from areas of inflamed and uninfamed intestinal biopsy tissue from patients participating in clinical trials which included periods of relapse and remission. This also afforded the opportunity to correlate change in *E. coli* numbers with histological findings and clinical state (chapter 7).

The crucial issues in the development of an assay for absolute quantification of *E. coli uidA* genes (and hence *E. coli* bacteria) using qPCR, were assay sensitivity, accurate DNA and gene copy quantification and the denominator used. In a previous study molecular quantification (218) of *uidA* genes in CD ileal and colonic tissue generated $0.5\text{--}5 \times 10^6$ *uidA* genes per million human cells in ileal biopsies and approximately 1.25×10^4 cells in colonic tissue, with 0.75×10^4 *uidA* genes in healthy tissue. The biopsies used in this work overall produced an average of 187000 18S rRNA genes per biopsy, or 937 human genomes (cells). From these figures, it is clear that the 18S rRNA assay used here was comfortably sensitive enough for CD biopsy samples, whilst the *uidA* gene assay was potentially more borderline, with a sensitivity of 1140 *uidA* gene copies. A further concern was that in one study looking at *uidA* genes in twins (226), levels of *E. coli* were significantly higher in patients with ileal CD than colonic CD but in support of this work however, the authors also conclude that when *E. coli* was detectable for either colonic or ileal disease it was

present at all areas of the gastrointestinal tract (ileum, ascending colon, transverse colon, descending colon and rectum), which is what was found here (chapter 7).

Reviewing the literature revealed that different studies use different denominators, which makes direct comparisons difficult for microbial ecology and absolute quantification. As an example, 16S rRNA gene copies have been expressed in a twin study per β -globin gene present (226), whereas in other studies *E.coli* quantities are expressed per 18S rRNA genomes (218). Whilst it is valid to use different or combined housekeeping genes even, the differing denominators for each gene quantity expressed make direct comparisons difficult, and ultimately one of the improvements of the assays used in this work are that they can be directly compared with other studies as each component examined was independently absolutely quantified.

The use of FISH analysis produces only semiquantitative answers. In one study (311) only categorical data was presented and the authors remarked that accurate quantification could not be achieved, particularly in the tissues with high bacterial concentration. Other authors have attempted to quantify *E. coli* and other bacterial numbers by specifying the percentage of biopsies examined with the specified bacterial species present within the tissue, without quantifying the actual quantity within each biopsy (200), also a categorical measure, in future work FISH could be of value in correlating absolute quantification, and defining spatial arrangement of bacteria.

Eventually, in view of these considerations, it was decided that the best solution was to aim for the maximum sensitivity possible for an absolute quantification assay (three bacteria), but that an acceptable sensitivity would approximate 1000 bacteria (per biopsy). The other targets were coefficient of

variation, or reproducibility <20%, and efficiency between 90-110%, error <0.2 and correlation coefficient >0.95. This was very difficult and pushed the limit of detection for qPCR to the extreme but all were eventually achieved.

The first quantification method resulted in a very poor standard curve, which may have been due to the crude method of DNA extraction used, or the fact that CFU counting can be inherently variable. The assay was clearly improved using an efficient commercial DNA extraction kit, but this still resulted in assay with poor efficiency, a key factor in PCR.

The switch to purely molecular techniques meant that there was removal of some of the variability seen when using methods involving CFU counting. The published genome size of *E. coli*, along with accurate quantification of extracted DNA using the Nanodrop TM, allowed good approximation of bacterial quantities, with a technique similar to that used previously (306). Unfortunately, this ultimately produced an assay with unacceptable error despite good efficiency and lower limits of detection (3530 copies)

Finally, the use of plasmid technology produced the definitive solution. The use of adapted plasmid DNA containing the *uidA* amplicon to transform cells was complicated, but meant that bacterial numbers could be quantified with great precision using these DNA standard templates and this approach was rewarded with a useable assay with appropriate sensitivity, efficiency, coefficient of variability and correlation coefficient. This technique is regarded as the gold standard currently (309;310) though it is in general vital to linearise plasmid DNA, as plasmid secondary and tertiary structure can preclude against sensitive target sequence annealing. This technique does have a drawback in that it can produce an over-estimation of gene numbers if the plasmid DNA is left in the supercoiled circular non-linear form,

because PCR amplification is less efficient when DNA is in this form (320) in the early stages of PCR amplification. For future work quantifying *E.coli* or broader bacterial groups, this would be the technique of choice.

Given its successful application to *uidA* quantification, it was appropriate to use the plasmid technology for the 16S rRNA gene assay. This reached the stage of development where a suitable clone had been produced for plasmid DNA and linearised, but when the plasmid DNA templates were used the qPCR did not reach acceptable performance statistics. Primer selection (as seen when different concentrations of primers were used with the *uidA* assays) and alteration of qPCR reaction conditions could improve this, though template quantity did not prove a crucial factor. Optimisation of this assay would provide useful denominator quantification of all bacteria present in biopsy DNA, and allow assessment of bacterial subset percentage changes in CD human tissue at different longitudinal timepoints and at different levels of disease activity, and would be an appropriate and expedient next step for analysis of microbiota in other biopsy samples, be they UC, CD, normal controls, or other gastrointestinal diseases. Both these and the *uidA* plasmid templates could also be used for microbial quantification for other diseases where solid organ tissue could be homogenised followed by DNA extraction and the techniques are not limited to gastrointestinal disease.

In contrast to the other assays the human 18S ribosomal RNA assay was straightforward, perhaps highlighting the importance of appropriate primers in qPCR, as this was a commercially developed kit with a probable high level of primer development. Its use as a housekeeping gene to standardise human tissue amounts was combined with standard biopsy mass measurement when CD biopsies were

processed, and concerns about its suitability as a denominator were not justified here, as both denominators yielded similar results. This assay could also be used in further quantitative studies of human tissue biopsies.

It was important to define a lower limit of detection for the assays and in this work this was simply defined as the limit where a fluorescence curve for a given template could be clearly and repeatedly differentiated from a PCR water control and also form part of a set of repeatedly useable templates that satisfied the statistical requirements for the PCR assay. The difficulties involved in looking at the lower limit of detection in qPCR are discussed in the literature. All appropriate statistics and methods are provided for this work so that these assays can be reproduced, consistent with good qPCR standards

When the qPCR standards were used successfully with human biopsy tissue for bacterial quantification, the main objectives of the first two chapters were achieved. The quantification was done in a robust and reproducible way. Though the numbers in both studies used as a source for biopsy data were small, nevertheless there were points of interest. The identification of high *uidA* gene quantities in the terminal ileal biopsy of a patient, with a macroscopically normal ileum but histological ileitis, and clinical markers of disease activity raises the possibility of whether bacterial *uidA* qPCR may eventually be used as an accessory predictive tool in pathology. The high quantities of the *uidA* gene in a lymphoid follicle suggests a possible role for these sorts of assays in the study of pathophysiological processes, given that this is the key tissue involved in pathophysiological bacterial translocation.

When the microbiota of patients in the plantain clinical trial were examined, an interesting finding was noted in the patients in sustained remission. The biopsies

were examined by the qPCR assay after pooling together mucus washings and biopsy material, and showed significantly reduced quantities of *uidA* genes. It will be even more informative when the clinical trial is unblinded and the effect of plantain fibre on both clinical relapse, and *uidA* gene quantity can be assessed in more detail, but certainly the fact that lower levels of the putative causative organism, *E. coli*, in CD, were noted in patients in remission was fascinating. and invites further work.

The use of qPCR to quantify *uidA* genes, and by proxy *E.coli* quantities in CD tissue from different lesions, in both periods of disease remission and relapse over time and also in small and large intestinal tissue at the same timepoint, may become a powerful tool. It could be used in examination of disease aetiology, and natural history, and perhaps could be used to quantify *E.coli* in clinical trials of antibiotics, as well as to measure the effects of steroids, immunomodulators, and anti-TNF agents on the quantities of *E.coli* in CD. It could also be used to quantify microbial changes with other clinical trial agents as well as these conventional drugs as a distinct endpoint in future clinical trials. It is also conceivable that microbiota quantification may have use as prognostic indicators of disease type, severity, or progress. As the AIEC group of bacteria become better characterised in terms of key genes involved in pathogenesis, for example the long polar fimbriae (*lpf*) gene (335), they will offer more specific targets for quantification. Subsequently the development of primers for qPCR of *E.coli* involved in CD, may be refined yet further.

In experiments evaluating the effects of drugs on *E.coli* replication within macrophages Ciprofloxacin was inhibitory and effective both at C_{\max} and 10% C_{\max} concentrations supporting previous work from this laboratory and also suggesting the importance of this antibiotic's use in clinical trials. The use of metronidazole as an

antibiotic to target the *E.coli* potentially relevant in CD pathogenesis is not supported by this *in vitro* work, as there is no evidence that it confers any additional benefit to intra-macrophage killing over ciprofloxacin used alone. Metronidazole has probable clinical efficacy in CD and has the support of meta-analysis (269), which may be due to the effective killing of anaerobic bacteria. These bacteria may infect tissue secondarily after mucosal ulceration has been initiated in response to other pathogenic agents. It also may have immunological effects, such as blockade of neutrophil recruitment which give it efficacy independent of its antibiotic effect (336), and it may be valuable for treatment of anaerobic infection in abscesses and perianal disease (356).

There is merit in clinical trials of combination antibiotics including ciprofloxacin for treatment of CD relapse. Further clinical trials of antibiotic combinations in CD which target *E.coli* specifically are warranted. Constructing these trials and selecting antibiotic combinations needs careful consideration of issues including side effect profiles, microbial resistance, and interactions with drugs used in maintenance therapy in CD.

The *in vitro* data examining the immunomodulators raise some interesting questions about the mode of action of some of the pharmacological agents used to treat IBD. Azathioprine and its metabolites 6-MP and 6-TG generally had some bacteriocidal effects when directly co-incubated with bacteria. Regarding the agents used in this work separately as an ‘antibiotic’ or as an ‘immunomodulator’ may also be an artificial simplification as they may possess properties of both, which has to some extent already been described. The mode of action of all the ‘immunomodulators’ to some extent seems to be related to altering sensitivity to reactive oxygen species (287;288), affecting the activity levels of iNOS (292), and

modulation of phagocytosis. This work prompts further examination of the mechanisms of action of azathioprine and its metabolites. In terms of effects on phagocytosis steroids had some interesting and unexpected effects. The suppression of intra-macrophage *E.coli* replication by hydrocortisone supports the data suggesting that steroids are pro-phagocytic (293), and that their effects on sepsis may be more related to their suppression of neutrophil recruitment in patients. It is probable that their effect on the body's response to sepsis may be mediated by blockade of the recruitment of neutrophils to a site of injury (360), which is mediated by IL-8 (202), or inhibition of macrophage recruitment (294), rather than by any direct effect on the ability of phagocytic leucocytes to kill bacteria. They were non bactericidal in direct co-incubation with bacteria, and this may be related to the timecourse they take to produce an effect rather than a genuine absence of effect. Alternatively it may be that steroids have differential effects on bacterial phagocytosis and killing at different timepoints. Many of the drugs used in this study demonstrate either a direct *E.coli* killing effect, or an indirect inhibition of the intra-macrophage survival of bacteria, and also already have proven clinical efficacy in CD management, either in maintaining remission or treating relapse. It is tempting to speculate that *E.coli* growth and replication, and its regulation is fundamental in CD pathogenesis, and that the evidence herein supports this. It would be useful to perform further experiments evaluating the effect on *E.coli* replication of other drugs or foods with proven CD efficacy, such as infliximab and enteral feeds, to delineate pathogenesis further. It is appropriate to quantify *E.coli* numbers in *in vitro* assays with the qPCR techniques developed in the first chapters of this work as they are highly specific and reproducible.

Work evaluating the effects of foodstuffs on diarrhoeal pathogens and AIEC adhesion to intestinal cells demonstrated that plantain NSP fibre has an inhibitory effect on the adhesion of AIEC, ETEC and *C. difficile* to intestinal cells. This was confirmed with both the *in vitro* modalities used, FACS and tissue culture, in the case of ETEC.

The absence of an inhibitory effect of NSP plantain fibre on the adherence of both typical and atypical EPEC bacteria to Caco2 cells has some biological plausibility, as the EPEC bacteria use a unique mechanism of interaction with host epithelial cells. Intimin receptor translocation onto the host cell functions as a receptor for its intimin ligand (253), and this may help the EPEC organism bypass the ‘mucosal blockade’, produced by plantain fibre by cross linkage of bacterial cell surface receptors, which will include lectin carbohydrate moieties. Soluble plantain fibre is a complex mixture of oligosaccharides, and any of these, perhaps particularly mannose, could interfere with the EPEC adhesion to the mucosal surface if the bacterium didn’t utilise these alternate host interaction strategies. Perhaps the atypical EPEC uses the proteins expressed from its mobility and resistance plasmids to avoid plantain cross linkage (252). The fact that both the negative and positive control experiments in the EPEC experiments gave the anticipated results supports the conclusion that this is a true result.

The non-significant inhibitory effects seen with apple pectin and oat NSP on ETEC inhibition need further development with dose response curves and further experimental repetition. It was of interest that the oat fibre did not stimulate bacterial adhesion to Caco2 cells as might be hypothesised from its’ stimulatory effects on NFkB in mice (363). Potentially and probably this results from different modulation of NFkB signalling in mice and humans. Also of note, the ETEC and *C.difficile*

adherence to Caco2 cells was inhibited by plantain NSP, and this supports the suggestion that toxin production is not the sole mechanism for disease activity, or at least that mucosal proximity is a factor in toxin production, as there certainly seemed to be ligands for mucosal adhesion that could be inhibited.

FACS results complemented tissue culture well results, but with some important exceptions. The ability to measure 10,000 cell-bacterial events in an experiment was a powerful technique, but the finding of a fluorescent signal generated by the plantain fibre in isolation was a significant drawback. This argues against future use of the assay in assessment of inhibition of bacterial adhesion by soluble fibres.

To further develop standard cell culture experiments they could be performed with qPCR techniques used to quantify final bacterial numbers to improve specificity, though for species not included in chapters six and seven, specific primers would need to be developed.

The potential for translating the results of these *in vitro* experiments into clinical trials investigating the prophylaxis of diarrhoeal pathogen infection, to complement the trials already underway investigating the use of plantain fibre as maintenance therapy in CD is clear (see chapter seven). A 5 g twice daily supplement of NSP plantain fibre would easily achieve intra-luminal concentrations demonstrated to have *in vitro* efficacy in this work (69;232). Diarrhoeal illness is a large burden worldwide, in terms of morbidity, mortality and cost, and antibiotics are often implicated as a cause of diarrhoeal illness. Additional strategies for prophylaxis of infective diarrhoea would augment current infectious disease prevention strategies. If dietary supplements are shown to be effective, it would result in lower incidence of multi-resistant bacteria and be very cost-effective. It could be of particular interest to

focus on at risk groups, for example hospitalised patients over the age of sixty five who are on antibiotics, African infants and children in areas of poor sanitation, or people travelling frequently to endemic areas. Many of these diarrhoeal pathogens can also trigger a relapse of UC or CD (47;224) so the benefit would not solely be confined to those patients at risk of diarrhoeal illness, but could also prevent CD relapses. The health and cost benefit from clinical trials of plantain and other foodstuffs is potentially vast, and again could use the highly developed qPCR assays from this work to quantify the microbiota as a genuine endpoint augmenting other conventional clinical measures.

The strengths of this study are that multiple elements of prevention of diarrhoeal illness were examined *in vitro* and *in vivo*. Attention was made to make *in vitro* experiments relevant clinically. The development of a precise method to quantify *E.coli* in tissue has far reaching potential in pathophysiological work. The weaknesses of the study include the relatively small number of patients recruited to the clinical trials, making demonstrations of differences in *E.coli* quantities between patient groups and between types of tissue difficult. In addition in the *in vitro* work, a limited number of *E.coli* strains were used due to time constraints.

Future work could include quantification of the AIEC pathotype in CD tissue using specific primers and quantification of total bacterial numbers would help delineate dysbiosis in CD tissue. Clarification of the differential cytokine expression by macrophages when incubated with current drugs used in CD therapy would be informative. The effect of drugs on labelled cellular components of the autophagy machinery, including the lysosome and phagosome should be addressed. Replication of other *E.coli* strains within macrophage tissue should be investigated, as should dose-response effects of apple-pectin and oat fibre on diarrhoeal pathogens.

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APPENDIX ONE : CLINICAL DATA FROM THE APHTHOUS ULCER STUDY

Information listed is in order of clinical indication, HBI score (all HBI's original scoring), bowel preparation taken, co-morbidities, medication, and CRP value. Cont = control patient, CD = Crohn's patient

PATIENT CODE	CLINICAL DETAILS
CONT1	Sigmoid polyp evaluated, HBI=0, kleenprep, Renal dialysis patient, Multiple medications – Quinine Clonazepam Sodium Bicarbonate Salmeterol Salbutamol Fludrocortisone Omeprazole Chlorpheniramine Aspirin Senna Frusemide Aranesp Atrovent Atorvastatin Calcichew Diltiazem retard , CRP -5
CONT2	Left iliac fossa and general abdominal pain associated with offensive stools and weight loss, HBI = 5, Moviprep, <i>Helicobacter pylori</i> positive and had bladder repair and hysterectomy for fibroid, Lansoprazole Movicol Senna and Metoclopramide, CRP < 5
CD1	CD surveillance - Crohn's colitis, HBI = 4, Moviprep, taking Salzopyrine, Vitamin B12 and Folate, CRP 14
CD2	Diarrhea mouth ulcers and arthralgia - new endoscopic diagnosis CD, HBI = 12, Moviprep, taking Microgynon, CRP 9
CD3	Previous right hemicolectomy symptomatic with diarrhea and abdominal pain, HBI = 13, Moviprep, Asthma and Obstructive sleep apnoea, Multiple medications - Pentasa Calcichew B ₁₂ Folic acid Salbutamol Seretide Tranexamic acid Cetirizine Cocodamol Terbutaline Salmeterol Carbocysteine Montelukast Bumetanide Lansoprazole Citalopram Domperidone Prednisolone Questran CRP 37

APPENDIX TWO : MACROSCOPIC AND MICROSCOPIC APPEARANCE OF TISSUE IN THE APHTHOUS ULCER STUDY

Macroscopic appearance of colonic ± ileal tissue in recruited patients

Cont = control, CD = CD patient.

PATIENT CODE	MACROSCOPIC APPEARANCE AT ENDOSCOPY
CONT1	Descending colon polyp. Nil else.
CONT2	Normal appearance
CD1	Some linear CD scars, otherwise quiescent appearance
CD2	Ileal and rectal CD - aphthous ulcers in the terminal ileum and splenic flexure ulceration typical of CD. Rectal inflammation
CD3	Quiescent CD, no ulcers or inflammation

Microscopic appearance of biopsy samples taken from large and small intestinal tissue of recruited patients

Cont = control, CD= CD patient.

PATIENT CODE	MICROSCOPIC APPEARANCE OF TISSUE
CONT1	Tubulovillous adenoma showing low grade dysplasia, excision complete
CONT2	Normal large bowel colonic mucosa
CD1	Large intestinal tissue from caecum to rectum showed mild crypt architectural distortion. Small numbers of muciphages in the recto-sigmoid. No active or significant inflammation
CD2	Focal chronic active colitis and ileitis with granuloma formation. Highly suspicious for CD. Ileal mucosa ulcerated with granulation tissue. Moderately severe chronic active inflammation seen. Transverse colon normal. Rectal mucosa focal chronic active colitis with small numbers of poorly formed granulomas within the lamina propria and submucosa.
CD3	Focal active inflammation in the ileum. Fragments of ileum show villous atrophy together with foci of ulceration and polymorphonuclear cells in the lamina propria and acute cryptitis. Normal large bowel mucosa

APPENDIX THREE : PHYSICAL CHARACTERISTICS OF BIOPSY SAMPLES FROM THE APHTHOUS ULCER STUDY

Physical characteristics of Small Intestinal biopsied

Mass and total quantity of DNA extracted from large and small intestinal tissues were quantified.

TI = terminal ileum, AU = aphthous ulcer, B = biopsy code no, LF = lymphoid follicle, SI = small intestine, serp = serpingous.

CODE	MASS/mg	DNA EXTRACTED ng/ μ L
CONT 2 TI	44.7	46.4
CONT 2 LF	7.2	31.2
CD1 B6 TI	64	129
CD2 SERP ULCER TI	36	25.4
CD2 AU	17.7	102
CD3 TI B	37.8	11.6
CD3 SI	44.8	14.5
CD3 TI B	56.1	14.2
CD1 B5 LF	32	46.3

Physical characteristics of Large Intestinal tissue biopsies

AC = ascending colon, SC = sigmoid colon, SF = splenic flexure, LI = non specific large intestinal sample, B = non specific colon sample

CODE	MASS/mg	DNA EXTRACTED ng/μL
CONT1 B1	81	14
CONT1 B2	20	107
CONT1 B3	12	43.7
CONT1 B4	113	55.5
CONT1 B5	27	37.6
CONT1 B6	27	101
CONT 1 B7	56	104
CONT 1 B8	96	109
CONT2 AC	55.5	27.1
CONT2 AC	70.3	n/a
CONT2 SC	59.3	11.6
CONT2 SC	59.2	2.19
CONT 2 SC	31.1	3.18
CONT2 CAECUM	112	33.5
CD1 B SF	63	87.9
CD1 B2	30	178
CD1 B3 SC	41	255
CD1 B4 TC	29	63.6
CD2 B SF	30.1	143
CD2 B SF ULCER	8.2	54.5
CD3 LI	24.5	18.6
CD3 LI	60.6	26.2
CD3 LI	59.7	9.28
CD3 LI	47.8	10.6

APPENDIX FOUR : QUANTITIES OF DNA EXTRACTED FROM MUCOSAL WASHINGS FROM SMALL AND LARGE INTESTINAL SAMPLES IN THE APHTHOUS ULCER STUDY

Small Intestinal Samples

Cont = control, CD = Crohn's patient, TI = terminal ileum, AU = aphthous ulcer, B = biopsy code no, LF = lymphoid follicle, SI = small intestine, serp = serpingous.

CODE	DNA QUANTITY ng/μL		CODE	DNA QUANTITY ng/μL
CONT 2 TI	5.72		CD3 SI	3.6
CONT 2 LF	n/a		CD3 TI B	1.6
CD1 B6 TI	10.9		CD1 B5 LF	5.7
CD2 SERP ULCER TI	9.3			
CD2 AU	90.2			
CD3 TI B	4.57			

Large Intestinal Samples

cont = control, CD = Crohn's patient AC = ascending colon, SC = sigmoid colon, SF = splenic flexure, LI = non specific large intestinal sample, B = non specific colon sample

CODE	DNA QUANTITY ng/μL		CODE	DNA QUANTITY ng/μL
CONT1 B1	6.2		CONT2 SC	11.6
CONT1 B2	14		CONT 2 CAECUM	2.50
CONT1 B3	7.9		CD1 B SF	85
CONT1 B4	10.4		CD1 B2	16.3
CONT1 B5	9		CD1 B3 SC	46.3
CONT1 B6	10.2		CD1 B4 TC	3.8
CONT1 B7	9.9		CD2 B SF	22.9
CONT1 B8	5.3		CD2 B SF ULCER	10.8
CONT2 AC	4.61		CD3 LI	3.6
CONT2 AC	4.1		CD3 LI	3.88
CONT2 SC	4.2		CD3 LI	2.62
CONT2 SC	3.6		CD3 LI	10.6

APPENDIX FIVE : PATIENTS STOPPING THE PLANTAIN CLINICAL TRIAL BEFORE TWELVE MONTHS

PATIENT CODE	REASON FOR TRIAL CESSATION
101	Patient withdrawal after one month. Near relapse CDAI = 213.5
201	Withdrawal after 5 months, with adverse effects (nausea and bloating). CDAI 76.8
202	Withdrawal after 6 months. CDAI rise of >100 to 141.
502	Relapse after 3 months. CDAI 363.7
603	Clinically relapsing at the time of withdrawal after 2 months. CDAI 311.3

APPENDIX SIX : CONSTITUENTS OF CULTURE MEDIA, AGAR, AND BUFFERS USED IN THIS THESIS

Culture Media

For Caco2 cells:

Dulbecco's modified Eagle's medium (DMEM), was supplemented with 10% vol/vol fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/mL streptomycin.

For J774-A1 macrophage cells

Cells were maintained in Roswell Park Memorial Institute RPMI-1640 medium, supplemented with 10% (v/v) FBS, 8 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

Agar used

LB agar consisted of the following components (per litre): Bacto-agar (20g), Yeast extract (5g), Bacto tryptone (10g) and Sodium Chloride (10g).

For liquid cultures, LB alone was used, omitting the Bacto-agar.

Amp-X-gal agar

500 ml of standard LB agar was prepared and autoclaved. After it was sufficiently cooled, but still in the liquid phase 400 ml was taken and 400 μ l Ampicillin (prepared at 100 mg/ml) added, together with 400 μ l X-Gal (80 mg/ml) and 200 μ l Isopropyl β -D-1-thiogalactopyranoside (IPTG, 100mM). This was mixed by swirling and agar plates were poured.

Buffer Composition

QIAprep Spin Miniprep (27104) buffer composition for plasmid DNA extraction

P1:

50 mM Tris-HCl pH 8.0

10 mM EDTA

100 μ g/ml RNaseA

P2:

200 mM NaOH

1% SDS

N3:

4.2 M Gu-HCl

0.9 M potassium acetate

(pH 4.8)

PE:

10 mM Tris-HCl (pH 7.5)

80% ethanol

Qiagen DNA mini-kit (51304) buffer composition**ATL:**

Proprietary chemical, contains:

EDTA

SDS

AL:

Proprietary chemical, contains:

Quanidium chloride

AW1:

Proprietary chemical, contains:

Quanidium chloride

AW2:

Proprietary chemical, composition unknown

Phosphate buffered saline composition (PBS):

Made up in 1 l sterile water

NaCl 8.01 g/l (137 mmol/l)

KCl 0.20 g/l (2.7 mmol/l)

Na₂HPO₄ – 2 H₂O 1.78 g/l (10 mmol/l)

KH₂PO₄ 0.27 g/l (2.0 mmol/l)

pH 7.4

Tris-acetate-EDTA (TAE):

Prepared as a 50X stock solution

242g Tris base in sterile water

57.1mL glacial acetic acid

100mL of 500mM EDTA (pH 8.0) solution

Final volume made up to 1 l with sterile water

(diluted 50:1 with sterile water to make a 1X working solution)

This 1X solution will contain 40mM Tris, 20mM acetic acid, and 1mM EDTA.

Tris-Borate-EDTA (TBE):

Prepared as 5X stock solution

54 g of Tris base

27.5 g of boric acid

20 ml of 0.5 M EDTA (pH 8.0)

TBE diluted to 1X or 0.5X with sterile water.

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